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COMPARATIVE BIOCHEMISTRY

Volume VI: Cells and Organisms

COMPARATIVE BIOCHEMISTRY

A Comprehensive Treatise

Volume I: Sources of Free Energy
Volume II: Free Energy and Biological Function
Volume III: Constituents of Life—Part A
Volume IV: Constituents of Life—Part B
Volume V: Constituents of Life—Part C
Volume VI: Cells and Organisms
Volume VII: Supplementary Volume

COMPARATIVE BIOCHEMISTRY

A Comprehensive Treatise

Edited by

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Volume VI

CELLS AND ORGANISMS

1964

ACADEMIC PRESS • New York and London

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ACADEMIC PRESS INC.
111 FIFTH AVENUE
NEW YORK 3, N. Y.

United Kingdom Edition
Published by
ACADEMIC PRESS INC. (LONDON) LTD.
BERKELEY SQUARE, LONDON W. 1

GX1 N60.6

Library of Congress Catalog Card Number 59–13830

CFTRI-MYSORE
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PRINTED IN THE UNITED STATES OF AMERICA

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PREFACE

The previous volumes of this treatise have provided a series of chapters on the comparative biochemistry of the biological transformations of energy (Vol. I and II) and of the biological transformations of matter (Vol. III to V).

Volume VI is devoted to chemical views on a number of biological concepts, all of them concerning the level of the complex architecture of cells and of organisms. Modern biochemistry has roots in organic and in physical chemistry, and these roots have been considered from the comparative standpoint in the previous volumes. But modern biochemistry also has roots in the biological sciences and these roots have given rise to an interest in the relationship between the structural composition, the distribution and metabolism of the chemical components of living organisms, on one hand, and the physiological, genetic, evolutionary and ecological properties of life, on the other.

The molecularization of biology, has fundamentally changed its outlook and the establishment of a causal link between biological concepts and aspects of the structure and function at the molecular level is rapidly growing. In some of these fields of studies, the viewpoint of the comparative biochemist is already bringing in new vistas and new perspectives, as will be made clear by the chapters of this volume.

M. Florkin
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October, 1963

CONTENTS

Contributors to Volume VI v

Preface vii

Contents to Volumes I, II, III, IV, V, and VII ix

Phylogenetic Charts xvii

1. The Biochemistry of Morphogenesis
Barbara E. Wright

I. Introduction 1
II. Bacterial Germination and Sporulation 4
III. Sporulation of the Cellular Slime Mold 19
IV. Seed Germination 30
V. Sea Urchin Egg Development 38
VI. Amphibian Differentiation 52
VII. Summary and Speculation 63
References 67

2. Comparative Aspects of Metabolic Control
Allan C. Wilson and Arthur B. Pardee

I. Introduction 73
II. Control of Enzyme Activity 74
III. Control of Enzyme Formation 81
IV. Activation of Enzymes 103
V. Discussion 105
References 107

3. The Biochemical Basis of Chemical Needs
Ernest Beerstecher, Jr.

I. Introduction 120
II. The Abiogenic Fulfillment of Biopoietic Demands 128
III. Requirements for Maintenance of the Fluid Medium 135
IV. The Nutritional Source of Free Energy 141
V. Nutritional Requirements for Structural Organization 148
VI. Requirements for the Catalysis of Organic Transformations . . . 169
VII. Summary and Conclusions 211
References 213

4. Biochemistry of Insect Metamorphosis
P. Karlson and C. E. Sekeris

I. Introduction 221
II. Morphology and Physiology 222
III. Respiration and Respiratory Enzymes 223
IV. Amino Acid and Protein Metabolism 227
V. Lipid and Carbohydrate Metabolism 231
VI. Phosphorus and Nucleic Acid Metabolism 232
VII. Special Metabolic Pathways Related to Metamorphosis 232

VIII. Interaction of Metamorphosis Hormone with Genetic Material . . 237
IX. General Conclusions 239
References 241

5. Hormones in Invertebrates

MANFRED GABE, PETER KARLSON, AND JEAN ROCHE

I. Introduction 246
II. Classification 247
III. Neurohumors 248
IV. Products of Neurosecretion 256
V. Hormones Elaborated by Tissues Other Than the Nervous System . . 270
VI. Pheromones 285
VII. Concluding Remarks 288
References 290

6. Protein Hormones in Vertebrates

ROGER ACHER

I. Introduction 299
II. Group of the Neurohypophyseal Hormones 302
III. Group of the Melanocorticotropic Hormones 316
IV. Group of the Insulins 330
V. Conclusions 339
References 340

7. Comparative Biochemistry of Digestive Mechanisms

H. J. VONK

I. Introduction 347
II. Digestion in General 348
III. Digestion in Special Physiological and Systematic Groups of Vertebrates 356
IV. Digestion in Invertebrates 377
V. Carnivorous Plants 390
VI. Concluding Remarks 390
References 392

8. Comparative Biochemistry of Detoxification

J. N. SMITH

I. Introduction 403
II. The Detoxication Mechanisms 404
III. Reaction Rates and Species Differences 441
IV. Developmental Aspects of Detoxication 444
V. Detoxication and the Adoption of a Terrestrial Habitat 447
References 448

AUTHOR INDEX 459

SUBJECT INDEX 492

COMPARATIVE BIOCHEMISTRY

A Comprehensive Treatise

Volume I: Sources of Free Energy

An Introduction to Comparative Biochemistry
    Marcel Florkin and Howard S. Mason

Thermodynamics of Living Systems
    Henry Eyring, Richard P. Boyce, and John D. Spikes

Comparative Mechanisms for Fatty Acid Oxidation
    P. K. Stumpf and G. A. Barber

Phosphoric Acid Anhydrides and Other Energy-Rich Compounds
    F. M. Huennekens and H. R. Whiteley

Onium Compounds and Their Biological Significance
    G. L. Cantoni

Phototropism and Phototaxis
    Kenneth V. Thimann and George M. Curry

The Distribution and Evolution of Visual Systems
    George Wald

Aerobic and Anaerobic Reactions of Inorganic Substances
    Claude Fromageot and Jacques C. Senez

Comparative Biochemistry of Glycolysis
    Ernest Bueding and Emmanuel Farber

Comparative Biochemistry of Electron Transport and Oxidative Phosphorylation
    Eric E. Conn

Utilization of Thermal Energy by Living Organisms
    Mary Belle Allen

Author Index—Subject Index.

Volume II: Free Energy and Biological Function

Free Energy and the Biosynthesis of Phosphates
M. R. Atkinson and R. K. Morton

Utilization of Free Energy for the Biosynthesis of Saccharides
Luis F. Leloir, Carlos E. Cardini, and Enrico Cabib

Comparative Biochemistry of Free Energy Utilization for the Biosynthesis of Peptides and Proteins
H. Chantrenne

Ammonia Metabolism and Urea Biosynthesis
Philip P. Cohen and George W. Brown, Jr.

Muscular Contraction
S. V. Perry

Other Mechanisms Producing Movements
Hartmut Hoffmann-Berling

Active Transport
B. Andersen and H. H. Ussing

Balance of Water, Electrolytes, and Nonelectrolytes
Freda Brown and W. D. Stein

The Mechanisms of Osmoregulation
J. Shaw

Nerve Conduction and Electrical Discharge
Michael A. Gerebtzoff and Ernest Schoffeniels

Bioluminescence
E. Newton Harvey

Author Index—Subject Index.

Volume III: Constituents of Life—Part A

The Comparative Aspects of Fatty Acid Occurrence and Distribution
F. B. SHORLAND

Sterols: Their Structure and Distribution
WERNER BERGMANN

Lipids: Steroid Metabolism
JAMES K. GRANT

Bile Salts: Structure, Distribution, and Possible Biological Significance as a Species Character
G. A. D. HASLEWOOD

Distribution of Phospholipids
JOHN C. DITTMER

The Metabolism of Phospholipids
R. M. C. DAWSON

Natural Monosaccharides and Oligosaccharides: Their Structures and Occurrence
D. J. BELL

Polysaccharidases
PETER BERNFELD

Saccharides: Alternate Routes of Metabolism
VERNON H. CHELDELIN, CHIH H. WANG, AND TSOO E. KING

Terpenoids: Structure and Distribution
W. SANDERMANN

Terpenoids: Metabolism
W. SANDERMANN

Quinones: Structure and Distribution
R. H. THOMSON

Melanins
R. H. THOMSON

Flavonoid Compounds
T. SWAIN AND E. C. BATE-SMITH

Author Index—Subject Index.

Volume IV: Constituents of Life—Part B

The Optical Asymmetry of Metabolites
T. L. V. Ulbricht

Cellulose, Starch, and Glycogen
J. S. Brimacombe and M. Stacey

The Biochemistry of Lignin Formation
F. F. Nord and Walter J. Schubert

Nucleic Acids
George Brawerman and Herman S. Shapiro

Protein Molecules: Intraspecific and Interspecific Variations
Alan Vegotsky and Sidney W. Fox

Metabolism of Aromatic Amino Acids
L. M. Henderson, R. K. Gholson, and C. E. Dalgliesh

Structural and Chemical Properties of Keratin-Forming Tissues
A. Gedeon Matoltsy

Sclerotization
M. G. M. Pryor

Silk and Other Cocoon Proteins
K. M. Rudall

Blood Coagulation
Charles Grégoire and Henry J. Tagnon

Metamorphosis and Biochemical Adaptation in Amphibia
Thomas Peter Bennett and Earl Frieden

Porphyrins: Structure, Distribution, and Metabolism
C. Rimington and G. Y. Kennedy

Pteridines: Structure and Metabolism
Hugh S. Forrest

Carotenoids: Structure, Distribution, and Function
T. W. Goodwin

Comparative Biochemistry of the Alkali Metals
H. B. Steinbach

Author Index—Subject Index

Volume V: Constituents of Life—Part C

Amino Acids: Structure and Distribution
B. Tschiersch and K. Mothes

Inorganic Nitrogen Compounds, Distribution and Metabolism
H. Takahashi, S. Taniguchi, and F. Egami

Acid Metabolism: The Citric Acid Cycle and Other Cycles
C. Lioret and A. Moyse

Comparative Biochemistry of Collagen
Jerome Gross

Photosynthesis
E. C. Wassink

Halides
J. Roche, M. Fontaine, and J. Leloup

Author Index—Subject Index.

Volume VII: Supplementary Volume

Expressions of the Pentose Phosphate Cycle
N. G. Pon

Chitin and Mucosubstances
P. W. Kent

Cellular Aspects of Active Transport
Ernest Schoffeniels

The Hormones and Behavior
William C. Young

The Comparative Biochemistry of Antibiosis and Antibiotics
Maurice Welsch

Author Index—Subject Index

Topical Subject Index Volumes I–VII

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[*] Most of the names refer to phyla, except in a few cases where some of the smaller taxonomic groups are shown. Capitalized names written across lines are groups including all forms above the name.

Note: Charts I, II, and III were prepared by Helen A. Stafford, Reed College, Portland, Oregon. For further information see "A Guide to the Nomenclature and Classification of Organisms," by Dr. Stafford, in Vol. I of this treatise.

CHART I
HYPOTHETICAL PHYLOGENETIC RELATIONSHIPS BETWEEN EXTANT MAJOR GROUPS OF ORGANISMS*

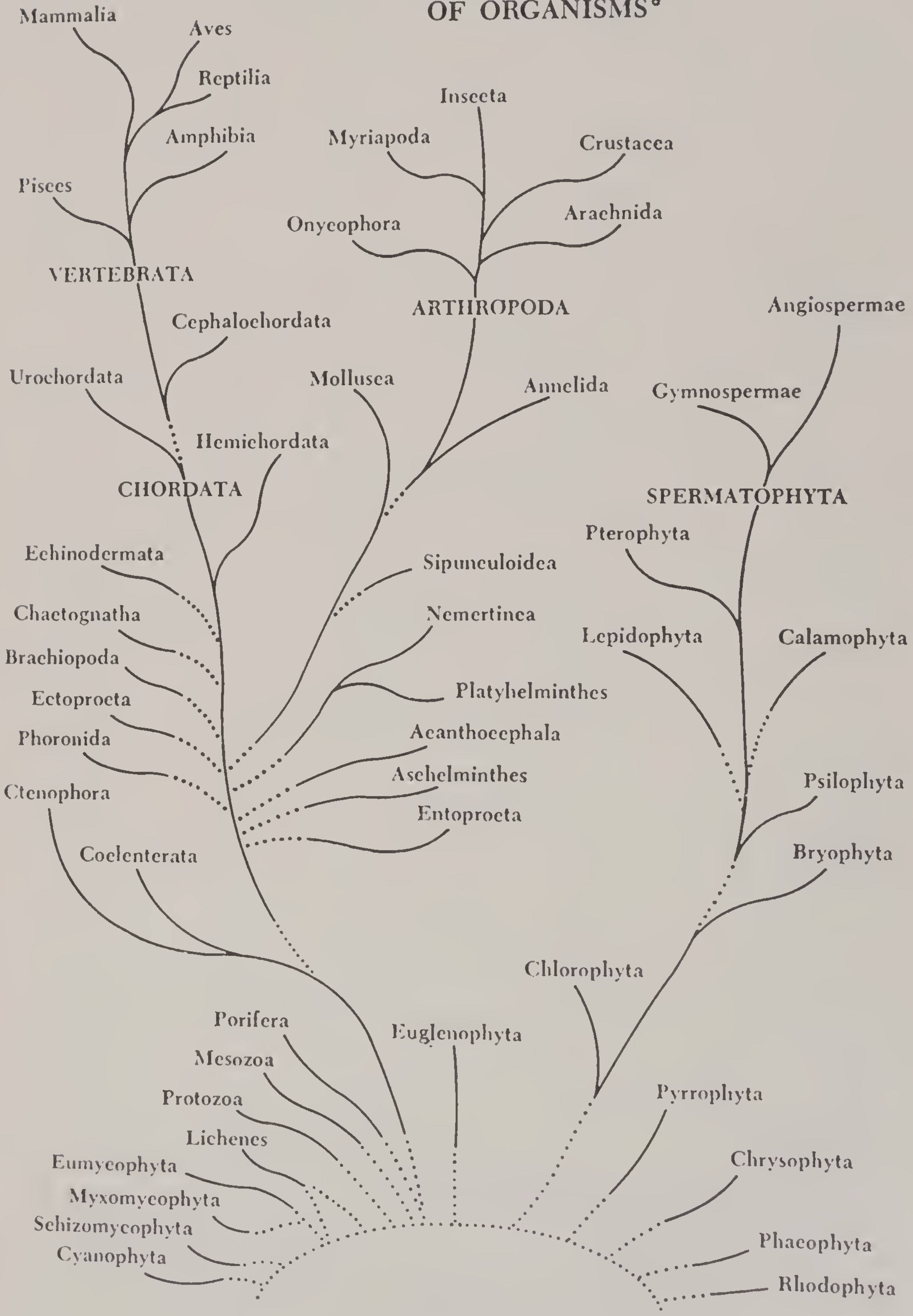

CHART II: ANIMAL KINGDOM

Divisions	Estimated Number of Species[d]	Taxonomic Classifications			
Protozoa (acellular animals)	15,000				
Mesozoa	—				
Porifera (sponges)	5,000				
Coelenterata (coelenterates)	10,000				Radiata
Ctenophora (comb jellies)	100				
Platyhelminthes (flat worms)	6,000	Acoelomates		Protostomia	Bilateria
Nemertinea (nemertine worms)	500				
Aschelminthes[a] Acanthocephala[a]	7,000	Pseudocoelomates			
Entoprocta[b] Ectoprocta[b] (moss animals)	3,000	Eucoelomates	Schizocoela		
Phoronida	15				
Brachiopoda (lamp shells)	120				
Mollusca (mollusks)	70,000				
Sipunculoidea	—				
Annelida[c] (segmented worms)	6,500				
Arthropoda (arthropods)	750,000				
Chaetognatha (arrow worms)	30		Enterocoela	Deuterostomia	
Echinodermata (echinoderms)	5,000				
Hemichordata Chordata (including vertebrates)	60,000				

---

[a] Includes Rotifera, Gastrotricha, Kinorhyncha, Nematoda, Nematomorpha, Priapuloidea. Formerly called Nemathelminthes.

[b] Formerly in Bryozoa.

[c] Includes Echiuroidea.

[d] Taken from "Handbook of Biological Data" (*4*), p. 533.

CHART III: PLANT KINGDOM

Divisions	Estimated Number of Species[d]	Major Synonymous Terms		
Euglenophyta (euglenoids)	340	**Algae**	**Thallophyta**	**Cryptogamia**
Chlorophyta (green algae)	5,700			
Pyrrophyta (cryptomonads, dinoflagellates)	1,000			
Chrysophyta (yellow green algae, diatoms)	5,700			
Phaeophyta (brown algae)	900			
Rhodophyta (red algae)	2,500			
Cyanophyta[a] (blue-green algae)	1,400			
Schizomycophyta[a] (bacteria)	1,300[e]	**Fungi**		
Myxomycophyta (slime molds)	430			
Eumycophyta (true fungi)	74,000			
Lichenes (lichens)	15,500			
Bryophyta (mosses and liverworts)	23,800		**Bryophyta**	
Psilophyta[b] (whisk ferns)	3	**Psilopsida**	**Tracheophyta**	
Calamophyta[b] (horsetails)	30	**Sphenopsida**		
Lepidophyta[b] (lycopods)	1,300	**Lycopsida**		
Pterophyta[b, c] (ferns)	10,000	**Pteropsida**		
Spermatophyta (seed plants)	201,000			**Phanerogamia**

---

[a] Sometimes grouped as Schizophyta.

[b] Formerly classed as Pteridophyta.

[c] Formerly classed as Filicineae in Pteropsida.

[d] Taken from "Handbook of Biological Data" (*4*), p. 533.

[e] There is much disagreement concerning designation of species here.

CHAPTER 1

The Biochemistry of Morphogenesis

BARBARA E. WRIGHT

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I. Introduction 1
II. Bacterial Germination and Sporulation 4
  A. The Initiation of Germination 5
  B. The Energy Requirement of Germination 5
  C. Electron Transport during Germination and Sporulation . . . 10
  D. The Initiation of Sporulation 11
  E. The Energy Requirement of Sporulation 12
  F. Mechanisms of Altered Enzyme Activities in Sporulation and Germination 14
III. Sporulation of the Cellular Slime Mold 19
  A. The Initiation of Multicellular Differentiation 20
  B. The Energy Requirement of Differentiation 20
  C. Mechanisms of Altered Enzyme Activities 27
IV. Seed Germination 30
  A. The Energy Requirement of Germination 30
  B. Mechanisms of Altered Enzyme Activities 36
V. Sea Urchin Egg Development 38
  A. The Energy Requirement of Differentiation 38
  B. The Double-Gradient Theory 45
  C. Mechanisms of Altered Enzyme Activities 47
VI. Amphibian Differentiation 52
  A. The Energy Requirement of Differentiation 52
  B. Mechanisms of Altered Enzyme Activities 57
VII. Summary and Speculation 63
  A. The Initiation and Control of Differentiation 63
  B. Mechanisms of Altered Enzyme Activities 64
  References 67

I. Introduction

Biochemical embryology is a young discipline in the sense that it is composed of many areas of research which have matured only recently: genetics, intermediary metabolism, hormone activation, enzyme induction, the chemistry of macromolecules, and "feedback" mechanisms controlling biosynthetic pathways. Thus we are just entering a period in which our knowledge is becoming detailed enough to allow a more rational approach to the biochemical basis of differentiation. Clearly, invisible biochemical changes must precede, and at least in part cause, the visible morphological changes usually referred to as differentiation.

The study of biochemical differentiation includes the problem of correlating enzymes and the products of enzyme action with morphological changes during development. It attempts to explain differentiation in terms of the gradual establishment of specific enzyme patterns and in terms of the sequential accumulation of substrates and products finally found in the characteristic cell types of the mature organism. There is abundant evidence for the existence of particular complements of enzymes in various tissues of an organism [see, for example, the enzyme analyses of Greenstein in various organs of the mouse (*1*) and Moog's data on enzyme differentiation in the chick (*2*)]. Studies such as these tell us something about the process of morphogenesis, and this knowledge inevitably focuses our attention on an even more basic problem of biochemical embryology: the underlying mechanisms *causing* the changes in various enzyme activities during the specialization of undifferentiated tissues. Whatever these mechanisms may be, they must share one common characteristic: that of a *differential* effect on the enzymatic potentialities of the cells in the developing organism. Whether it be differential repression or activation, there must arise local imbalances in presumptive cell types such that particular enzymes characteristic of one tissue become active and others are in effect repressed. Thus growth in the sense of simple cell duplication at optimal rates is opposed to differentiation, which requires that a cell gain the ability to give rise to progeny unlike itself.

Biochemical differentiation will be broadly defined as the intrinsic development of alterations in the enzymatic activities of a cell. This definition stresses biochemical alterations primarily initiated by the normal events accompanying growth and differentiation. At least three possible mechanisms (*a–c*) leading to heterogeneous cell types with respect to enzyme activities could involve alterations in the activity but not the concentration of an enzyme protein: (*a*) differential substrate or coenzyme availability; (*b*) differential activation of preformed, inactive enzyme protein; (*c*) differential suppression of enzyme activity by end products of the reaction or by hormones, for example.

At least four possible mechanisms (*d–g*) which could result in altered enzyme activities during development involve alterations in the concentration of an enzyme protein: (*d*) differential destruction of an enzyme; (*e*) differential enzyme stabilization (and hence accumulation), for example, by substrates; (*f*) differential rate of enzyme synthesis due to the activity of inducers or templates; (*g*) differential rate of enzyme synthesis due, for example, to suppression by end products.

If one includes both *in vivo* and *in vitro* evidence, it will be seen that the literature provides examples of mechanisms *a–e* in differentiating systems. Only (*f*) and (*g*) involve a change in the rate of synthesis of an enzyme. Although these two types of enzyme regulation undoubtedly occur, they are very difficult to demonstrate. Probably many of these mechanisms coexist and interreact, thereby contributing to the amazingly stable and reproducible process of differentiation.

It is not the purpose of this chapter to supplement Needham's thorough work (3) by an exhaustive review of the more recent literature. Rather, particular emphasis will be placed on more recent data considered relevant to an understanding of the mechanisms responsible for altered enzymatic activities associated with differentiation. Insofar as it is possible to do so, we shall bring together certain common denominators (e.g., alterations in respiration, enzyme activities, etc.) in the biochemistry of representative types of differentiating systems. These biochemical events will be compared to each other and to similar changes found in systems not complicated by complex multicellular development. Selected biochemical transformations in unicellular organisms will also be considered, particularly insofar as similar changes appear to be involved in the developmental processes of multicellular organisms.

Five systems have been selected as focal points for purposes of discussion and comparison: (*a*) germination and sporulation in bacteria; (*b*) multicellular differentiation of cellular slime molds; (*c*) seed germination; (*d*) sea urchin egg development; (*e*) amphibian development. Very relevant work in other organisms such as the water mold, the chicken egg, and the diapausing insect should have been included. Unfortunately, owing to limitations of time and space, only occasional reference can be made to these interesting investigations.

It may appear that disproportionate space is devoted to the germination of a bacterial spore compared to the biochemical changes accompanying the differentiation of an amphibian. In the former case, however, there is one cell type instead of many, a fact that renders an analysis of the biochemical mechanisms involved feasible. Even if it were possible to analyze the biochemical transformations of a single cell type in the amphibian, it would also be necessary to understand the direct and indirect interaction of many types of cells, not to mention possible complexities such as hormone action. Needless to say, the interaction between cell types cannot be studied in a unicellular organism. In our present state of ignorance about the biochemistry of morphogenesis, however, useful insight into fundamental mechanisms responsible for cellular transformations may best be sought through a detailed understanding of organisms with one or only a few cell types. Whether biochemical mechanisms elucidated in such simple systems will eventually be applicable to the chicken egg remains to be seen. The facts of comparative biochemistry suggest that they will.

Undoubtedly fundamental to the remarkable precision of differentiation are regulatory mechanisms. These may range in complexity from the inhibition of a specific enzyme by some end product of its activity to the mutual inhibition (or stimulation) of the products of one cell type by another. Such interactions could in turn control the respective biochemical and morphological roles of various cell types in a complex organism. Since, in the course of evolution, the multicellular arose from the unicellular organism, it is not unlikely that regulatory mechanisms operating in microorganisms not only are present in multicellular organisms but form the basis for more complex regulatory systems such as those under hormonal control. One type of regulatory mechanism of basic importance to differentiating systems are those concerned with the balance between anaerobic and aerobic metabolism. As cells begin to develop into various presumptive types and acquire their characteristic synthetic abilities, respiration frequently increases, perhaps correlated with the added energy demands of specialization. It is of particular interest in this connection that, when differentiated cells lose their unique organization and function, as in malignancy or tissue culture, their growth becomes uncontrolled and less aerobic.

Thus, apparently similar biochemical phenomena of development will be drawn together from creatures of greater or lesser complexity. These generalizations will probably in part be superficial or even invalid. Nevertheless, they may be provocative and are offered in the hope that such suggestions and speculations may help in posing fundamental questions susceptible of critical analysis.

II. Bacterial Germination and Sporulation

The germination of a bacterial spore represents perhaps the simplest and hence most analyzable model system for studying biochemical differentiation. This is borne out by the fact, which will become evident, that many biochemical alterations observed during germination and sporulation are comparable to changes that occur during the differentiation of more complex organisms.

A. The Initiation of Germination

A spore can germinate in a few seconds under optimal conditions; spore rupture can be followed by a striking difference in optical density or heat resistance associated with the new vegetative cells. The stimuli for this remarkable transformation usually include an amino acid as a source of nitrogen and carbon, as well as some precursor of nucleic acids (*4*). In the germination of *Bacillus cereus* spores, L-alanine can be replaced by glucose or spared by lactate or pyruvate (*5*). Intermediates of hexose metabolism, and amino acids giving rise to such compounds, are key stimulants of the germination process. An amino acid may be more effective than pyruvate, for example. However, lowering the pH can render pyruvate and acetate effective stimulants, a fact strongly suggesting that negative results with such intermediates are due to the impermeability of the dissociated acid. Heat shock enhances the effect of germinating agents, presumably because of increased permeability of the spore wall. It has also been suggested that the enhanced activity of proteolytic enzymes at elevated temperatures may be an important consequence of heat shock (*6*). The aging of spores speeds up germination, as well as making the nutrient requirements for activation less exacting. Apparently aging is associated with an autolytic process which in turn is responsible for the endogenous liberation of germination stimulants. For example, L-alanine has been identified in the washings of aged, but not of fresh, spores (*5*).

The dormancy and resistance of a bacterial spore has been attributed largely to its anhydrous state, ensured by an impermeable waterproof barrier (*7, 8*). In this view hydration of the spore, initiated by mechanical abrasion or physiological germinants, is the primary event resulting in germination and loss of heat resistance.

B. The Energy Requirement of Germination

1. *Alterations in Respiration*

An important characteristic of a germinating spore is its altered rate of respiration. This is illustrated in Fig. 1, which shows the oxygen consumption of heat-shocked, germinating *Bacillus megaterium* spores in the presence of various stimulants. An initial burst of respiration accompanies germination. The subsequent plateau is associated with swelling of the cells prior to their actual multiplication, which is correlated with a new increase in the rate of respiration as well as a requirement for inorganic sulfate (*9*).

A more critical look at the intimate correlation between oxygen

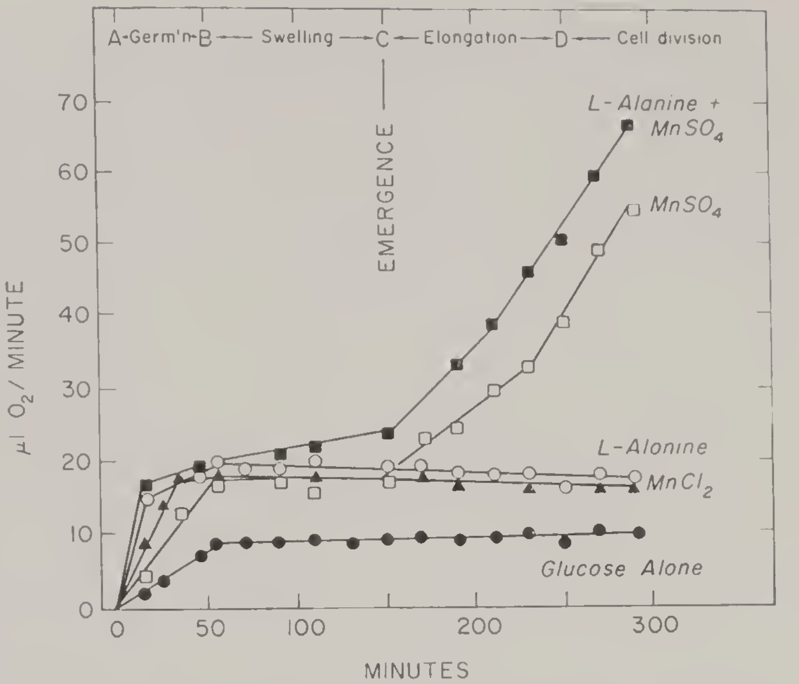

Fig. 1. Respiration of *Bacillus megaterium* during germination. From Levinson and Sevag (*9*).

consumption and germination can be seen in Fig. 2. Respiration and percentage of germination are plotted as a function of manganese concentration. The reaction period was 140 minutes at 30° C. (*10*).

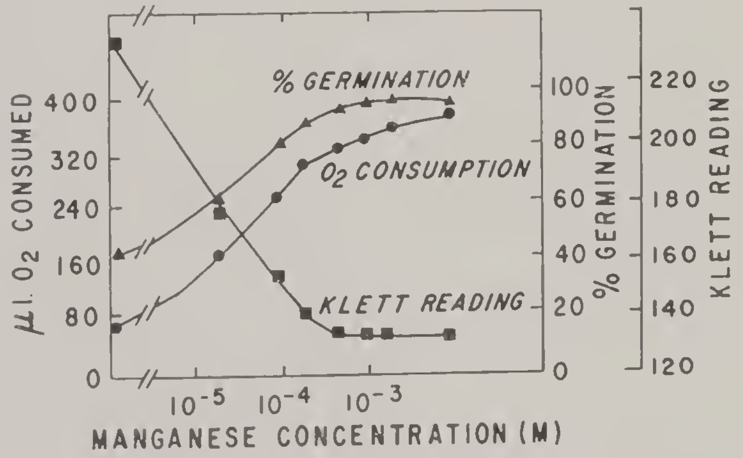

Fig. 2. Respiration during germination as a function of manganese concentration. From Levinson and Sevag (*10*).

The role of manganese in stimulating germination will be discussed in more detail below; the evidence suggests that it activates certain proteolytic enzymes which in turn liberate germination stimulants. The indirect action of manganese compared to alanine is immediately suggested by the fact that its action is less rapid. The maximal degree of germination can be reached in 5 or 10 minutes in the presence of alanine, whereas the same degree of germination can take as long as 1.5 hours with manganese (*6*).

Since alanine alone can so rapidly stimulate both germination and respiration, the question arises as to the possible dependence of germination on alanine oxidation. Detailed studies have in fact demonstrated that alanine is readily converted to pyruvate and ammonia by heat-activated spores and that oxidative metabolism of pyruvate is necessary to the germination process. The experiment presented in Table I employed a specific inhibitor of the oxidative decarboxylation of pyruvate: bis-1,3-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine (BEP). The specificity of its action toward pyruvate oxidation is shown, as well as a partial reversal of the inhibition by cocarboxylase and thiamine (*11*).

TABLE I
INHIBITION OF PYRUVATE OXIDATION BY BEP[a]

	O_2 uptake (microliters per 60 min.) with	
	Glucose	Pyruvate
Control	160	56
BEP	162	0
BEP + cocarboxylase	—	49
BEP + thiamine	—	18

[a] From Halvorson and Church (*11*).

Table II clearly demonstrates the obligatory coupling of pyruvate metabolism to germination in spores of *Bacillus cereus* strain T. Partial reversal of the inhibition of pyruvate utilization also results in a partial reversal of the inhibition of germination (*11*). It is evident that alanine oxidation is in fact essential to the mechanisms of germination in *B. cereus.*

TABLE II
THE DEPENDENCE OF GERMINATION ON PYRUVATE OXIDATION[a]

Substrate	NH_3 (μmoles)	Pyruvate (μmoles)	Germination (%)
None	0	0	0
Glycine	0	0	0
Phenylalanine	0	0	0
L-Alanine	5.0	0	100
L-Alanine + BEP	5.0	5.2	3
L-Alanine + BEP + thiamine	4.6	2.5	41

[a] From Halvorson and Church (*11*).

Another germination stimulant, adenosine, is at least partially degraded through the action of adenosine deaminase and ribosidase (*12*, *13*). A number of carbohydrates which can serve as germinating agents are also actively oxidized. It thus appears probable that most of these stimulants directly or indirectly serve as energy donors, as well as giving rise to essential intermediates.

2. *Utilization of Endogenous Reserves*

Although exogenous alanine is preferentially utilized early in germination, it is of significance that most of the products resulting from the action of alanine on spores arise from endogenous sources. In the presence of L-alanine, ammonia is released rapidly by heat-activated spores. However, heat activation alone also stimulates the release of ammonia from endogenous substrates. Attempts to correlate alanine disappearance with ammonia released revealed that more ammonia was formed than could be accounted for by alanine utilization (Fig. 3) (*14*).

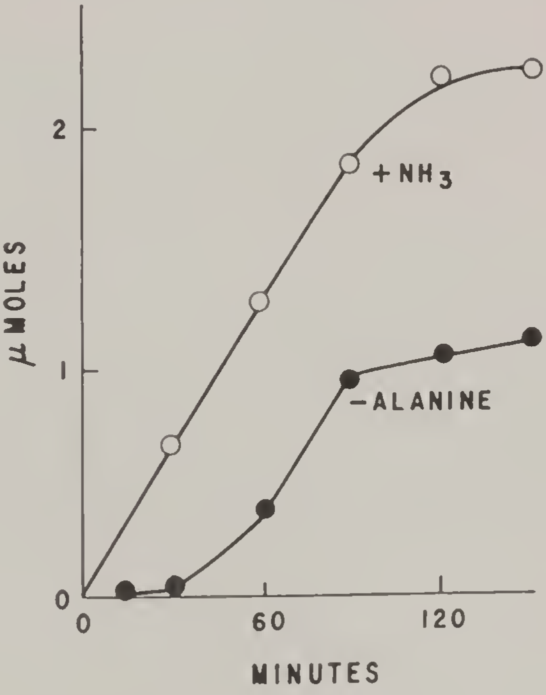

FIG. 3. Ammonia release and alanine utilization during alanine activation of *Bacillus cereus* spores. From O'Connor and Halvorson (*14*).

A further analysis using N^{15}-labeled alanine revealed that later in germination only 10% of the NH_3 was derived from exogenous alanine. Similar experiments in which labeled pyruvate was recovered from C^{14}-labeled alanine indicated that almost 90% of the pyruvate was produced from endogenous sources. Thus it appears that alanine stimulation has "sparked" the utilization of endogenous reserve materials. This important observation will be discussed in the section (F) dealing with the mechanisms of altered enzyme activity.

By a somewhat more obvious process, manganese activation also stimulates spores to degrade their macromolecules. Proteolytic activity against gelatin, egg albumin, and the proteins of spore homogenates has been shown to be manganese stimulated. Cobalt and zinc, which are peptidase activators, also stimulate spores of *B. megaterium* to germinate (*6*). Furthermore, a number of proteolytic enzymes are most active at elevated temperatures, which may be quite relevant to the phenomenon of heat activation. Dialyzable materials from spore extracts have been shown to stimulate respiration and germination; the dialyzate contained a number of amino acids, among which were alanine and glutamate in relatively high concentration (*6*).

In vivo evidence for the release of endogenous amino acids during germination was obtained by the use of spores of *B. cereus* (*15*). The spores were labeled by previous growth in $S^{35}O_4$, and their free S^{35}-methionine content was determined before and after partial germination (loss of heat resistance). After incubation for 50 minutes in a germination solution, 24 times as much free methionine existed as in ungerminated spores, and 14 times as much as in controls incubated in plain buffer. These experiments demonstrate that free amino acids are formed from spore proteins very early in the germination process. In this connection, a decrease in protein-bound phosphorus has also been demonstrated (*16*).

Recent studies in *Aspergillus niger* have demonstrated the existence in spores of a store of reserve phosphates, such as polyphosphate and phospholipid (*17*). During early germination these "phosphagens" decrease in amount, and various nucleotides and sugar phosphates increase in the acid-soluble fraction. Adenosine triphosphate (ATP) and glycerol phosphate appear in significant amounts, a fact suggesting that the phosphagens serve as an energy source during early germination. The interesting observation was made that polyphosphate utilization is apparently linked to nitrogen metabolism insofar as some nitrogenous metabolite(s) may be necessary as a phosphate acceptor in the utilization of phosphogen. Another "trigger" mechanism is thus suggested in the utilization of endogenous nutrients.

In addition to NH_3 and CO_2, a number of degradation products are excreted from *Bacillus* spores during germination. This "germination exudate" consists of calcium dipicolinate, a nondialyzable peptide containing diaminopimelic acid, small amounts of protein, a number of low molecular weight peptides and free amino acids (*7*). Thus, germination is accompanied by a 30% loss in dry weight, over half of which is due to the loss of dipicolinate. Extracts of spores have been found to contain a lytic system capable of releasing the diaminopimelic peptide from an insoluble fraction of vegetative cells (*18*).

C. Electron Transport during Germination and Sporulation

As noted previously, the respiration of spores is very low, rising abruptly at germination and again during outgrowth of the vegetative cells (Fig. 1). The cytochrome content in spores of *Bacillus subtilis* was found to be only 6% that of the vegetative cells (*19, 20*). Correlated with the increased importance of a cytochrome system during development is an increased sensitivity to cyanide (*21, 22*). The respiration of spores, as well as the oxidation of glucose by spores, is cyanide insensitive. Fully developed vegetative cells are more sensitive to cyanide than newly germinated cells. A plausible explanation for this situation is the presence of an excess of heat-stable cytochrome oxidase (*23*) over cytochrome c, which would represent the limiting step in the respiration chain. Under such circumstances, the inhibition of a large fraction of the cytochrome oxidase present might not result in an inhibited respiration. Such an explanation has been proposed for diapausing insects to explain alterations in cyanide sensitivity (*24*).

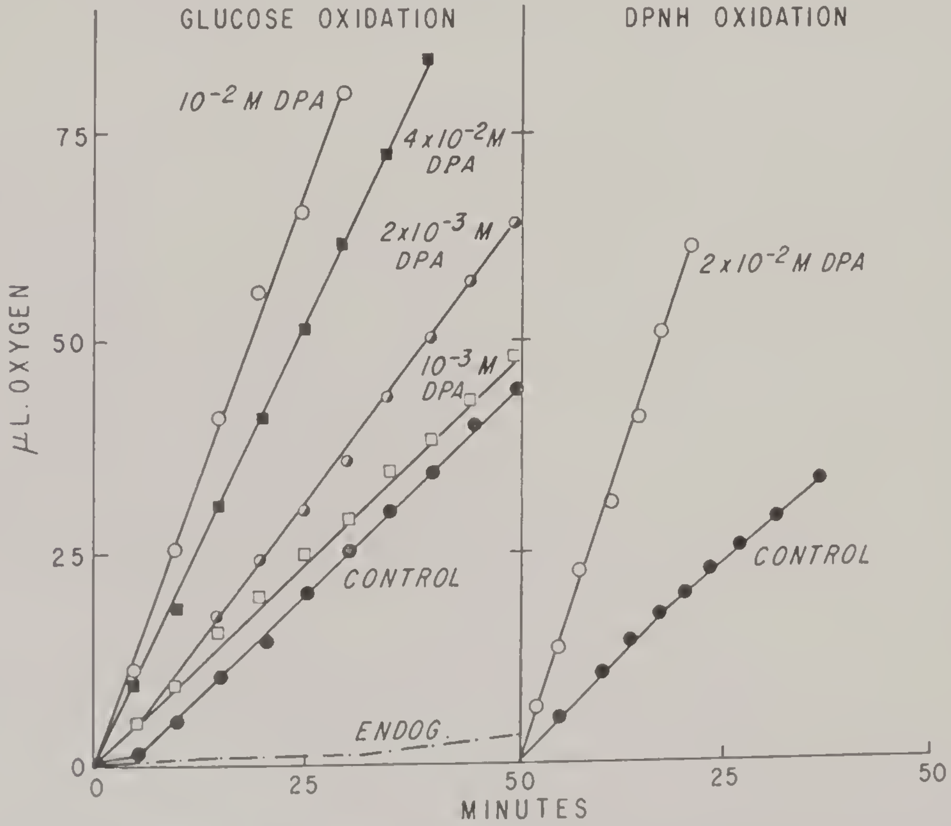

FIG. 4. Stimulation of oxygen consumption by dipicolinic acid (DPA) in the presence of spore extracts. From Doi and Halvorson (*26*).

Another explanation for the lack of cyanide sensitivity in spores is of course the possibility of a functioning, non-cytochrome-linked terminal oxidase such as a flavoprotein. In fact, spore extracts contain both a free and combined flavin-adenine dinucleotide as well as a diaphorase-like system (22). A critical role for a flavin-linked oxidase in spores has recently been made very probable by some interesting studies on the function of dipicolinic acid during germination.

As mentioned previously, germination is accompanied by the release of large amounts of dipicolinic acid (DPA). The presence of DPA-stimulated oxygen uptake from both glucose and reduced diphosphopyridine nucleotide (DPNH) in the presence of spore extracts is shown in Fig. 4 (25). Further work demonstrated that a particulate and a soluble DPNH oxidase could be separated in extracts of both the spores and vegetative cells of *B. cereus* (26). The particulate oxidase, coupled to a cytochrome system, was 300 times more active in the cells than in the spores, whereas the soluble oxidase was 4–5 times more active in the spores. This latter oxidase was found to be dependent on flavin mononucleotide and to some extent on DPA. The data strongly suggest that the principal electron transport system in spores is via a soluble DPNH oxidase which can utilize DPN as an electron acceptor. It is tempting to conclude that DPA release during germination promotes respiration, in part, by direct stimulation of soluble DPNH oxidase, thus enhancing the oxidation of endogenous substrates such as alanine (27).

D. The Initiation of Sporulation

Considering that germination marks the beginning of vigorous growth and multiplication, and that sporulation represents the end of growth and a period of metabolic dormancy, it is really surprising how much the two processes have in common. Of course, both phenomena involve the differentiation of one cell type into another. Although initiated under quite different circumstances, both germination and sporulation are accompanied by the degradation of endogenous macromolecules and the subsequent formation of either vegetative cells or spores.

Under normal circumstances, spore formation begins when vegetative growth ceases. It has been suggested (28) that the primary event in the metamorphosis of a vegetative cell to a spore may be a dissociation of the dependence of endergonic reactions on an exogenous energy source. In this connection it is of interest that glucose, in the absense of growth, can retard sporulation (28). Normal sporulation was 90% complete after the preparation had been shaken for 11 hours in water (see Fig. 5). The addition of 0.1% glucose between the 1st and 5th hours completely suppressed sporulation. Added at the 6th, 7th, or 8th hours, glucose was progressively less inhibitory; it was totally ineffective if added after the 9th hour. By this time, the cells are clearly committed to spore formation, even in the presence of fresh nutrients. The glucose inhibition could be interpreted as feedback inhibition or repression of enzymes necessary to the sporulation process.

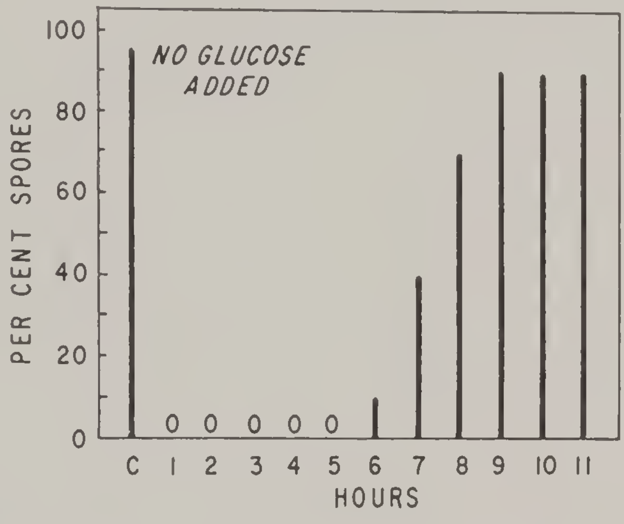

FIG. 5. The inhibition of sporulation by glucose. From Foster (28).

When the exogenous energy-yielding substrate is consumed, or when its utilization is in some way impaired, endogenous high molecular weight compounds are degraded and used as sources of energy and building blocks for the synthesis of substances unique to spores.

E. THE ENERGY REQUIREMENT OF SPORULATION

1. *Alterations in Respiration*

In common with germination, the sporulation process is immediately preceded by a burst of respiratory activity. The oxygen demand of a culture of *Bacillus cereus* has been measured with a polarograph during growth and sporulation, with the interesting results summarized in Fig. 6 (29). A peak in oxygen demand was reached at the stage of maximum cell number (2¼ hours). Respiration then decreased and the decrease was followed by a sharp rise to a level higher than that obtained at the peak of the growth phase. This final burst of respiration marked the beginning of sporulation. A possible explanation for these alterations in oxygen demand was suggested by the pH curve, also shown in Fig. 6. If organic acids accumulated from the incomplete oxidation of glucose, and if these acids were subsequently oxidized and disappeared from the medium, the observed alterations in pH and respiration could be explained.

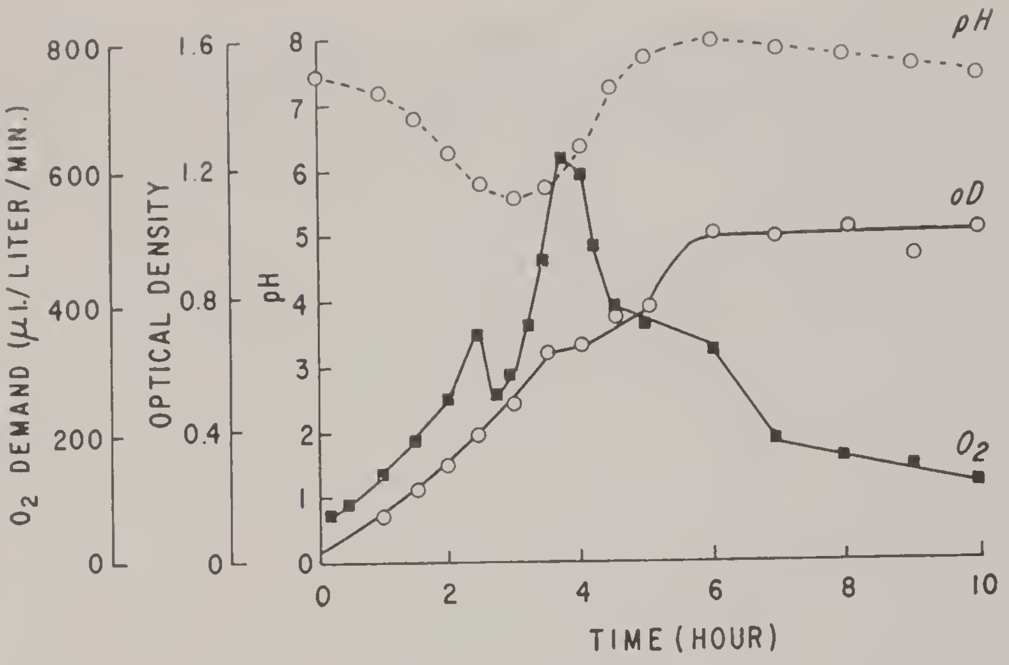

FIG. 6. Respiration during germination of *Bacillus cereus*. From Halvorson (*29*).

In fact, at the time of glucose exhaustion from the medium, there occurred a maximal accumulation of acetic and pyruvic acids, correlated with the lowest value in the pH curve, the cessation of growth, and the initiation of sporulation. As spore formation proceeded, acetic acid disappeared from the medium. The activation of a system oxidizing poly-β-hydroxybutyric acid has also been shown to occur just prior to the sporulation process (*30*). It is tempting to speculate that, in a manner perhaps comparable to alanine activation of germination, the metabolism of these compounds may in part be necessary as an energy and carbon source for the initiation of spore formation. It should be pointed out, however, that anaerobic bacteria also sporulate and that no burst of respiration occurs during slime mold sporulation. Of course, fermentative activity may replace respiration as an energy source in these cases.

2. *Utilization of Endogenous Reserves*

The degradation of endogenous materials in the absence of active growth is a characteristic of microorganisms and is not unique to systems undergoing biochemical differentiation. The factor that causes growth to stop is of secondary importance; deficiencies in an amino acid, nitrogen, or phosphorus can stimulate ribonucleic acid (RNA) and protein degradation without lag (*31*). The available evidence suggests that proteolytic enzymes may exist in an inactive (particle-bound?) state during growth and become activated in some way under conditions of starvation, perhaps as a result of ribosome disintegration. An important cause of endogenous protein degradation may well be a deficiency, brought about by starvation, in the intracellular concentration of the normal "feedback" inhibitors of proteolysis. Ammonium ions inhibit protein degradation, and a decrease in the level of free amino acids apparently stimulates proteolytic activity. Thus starvation does not appear to induce the formation of proteases (which do not increase in concentration), but rather to activate pre-existent systems (*32*, *33*). During spore formation the degradative metabolic events initiated by starvation are accompanied by the synthesis of specific substances characteristic of the spore, such as dipicolinic acid and a cystine-rich protein structure (*34*). As in the case of starving *Escherichia coli* cells, the free amino acid pool is largely depleted during sporulation of *Bacillus cereus* var. *mycoides* (*35*). By the 8th hour of incubation in water, at least four amino acids are reduced to roughly 30% of their initial level in vegetative cells. Endogenous protein utilization also occurs during sporulation, and in some cases is apparently brought about by a Mn^{2+}-stimulated proteinase (*36*). The requirement of manganese as a stimulant for sporulation (*37*), as well as its role in activating a proteinase, is reminiscent of the comparable role of manganese in germination.

Cellular protein breakdown during sporulation in water has been studied by following the liberation of S^{35}-methionine from labeled endogenous protein (*35*). A peak in the release of soluble label was found to occur at about 3–4 hours, under conditions in which sporulation occurred between the 9th and 12th hours. Concomitant with an increase in the soluble fraction was a decrease in the insoluble fraction. That the amino acids thus made available were reutilized by the cell was demonstrated by the incorporation of S^{35}-methionine into protein. In this case, the maximum rate of methionine fixation into protein occurred at the 6th to 8th hour, i.e., somewhat after the peak in protein degradation. As will be seen below, very comparable results have been obtained during the differentiation process resulting in the sporulation of slime molds. Further analysis in this system, however, revealed that an apparent increase in the rate of protein synthesis was actually due to an enhanced rate of exchange of exogenous labeled methionine with endogenous unlabeled methionine (*38*). Whether this situation exists during bacterial sporulation is not known.

F. Mechanisms of Altered Enzyme Activities in Sporulation and Germination

1. *Evidence for Various Enzyme Activities*

There are groups of enzymes which are heat stable and active in spores, and others which are "dormant" but can be activated by heat or by nutrients. A systematic listing of enzymes falling into various categories can be seen elsewhere (*4*). We shall be concerned in this discussion primarily with a few representative enzyme systems, the characteristics of which suggest possible mechanisms for the alteration of enzymatic activities.

In view of the fact that sporulation can proceed under starvation conditions and is accompanied by a depletion of endogenous oxidizable substrates, it is not surprising that mature spores exhibit a low rate of respiration and metabolism. The interesting question concerns the fate of various enzymes under such circumstances. For some time spores were considered to be virtually devoid of enzymatic activity. Recently it has been found that ungerminated spores may contain most of the enzymes found in their vegetative forms, and vice versa. Exceptions would include "specialized" enzymes such as those associated with DPA synthesis.

Negative results were obtained initially in attempts to observe oxidative activity toward citric acid cycle intermediates in spore extracts (*39*). In later studies, however, such activities were detected if the reaction mixture were sparked by the addition of oxalacetate. Presumably there was an insufficient endogenous supply of this substrate catalyst in spore extracts (*4*). The glucose-oxidizing system was initially thought to be absent *in vivo* (*40*). It was subsequently found, however, that the addition of alanine to a spore suspension could "activate" the oxidation of glucose (*41*). The absence of a functional Embden-Meyerhof pathway was deduced from the inability of spore extracts to ferment glucose or phosphorylated derivatives thereof. Furthermore, hexokinase, phosphohexokinase, aldolase, and triosephosphate dehydrogenase could not be detected (*4*). Recent evidence, on the other hand, based on the presence of various enzymes in spore extracts and on experiments using isotopic techniques in intact spores, indicates that in fact the Embden-Meyerhof pathway must be a major route of glucose catabolism in spores of aerobic bacilli (*42, 43*). The failure to obtain evidence for this pathway *in vitro* was in part due to enzyme instability. It is now known that hexokinase can be routinely detected only if cells are disrupted in the presence of 1% glucose. As will be seen later, *in vitro* substrate protection has also been observed at certain developmental stages of the cellular slime mold. In view of these difficulties in detecting enzyme activities, it is clear that data indicating the absence of an enzyme should be accepted with reservations.

2. *Release of Preformed Enzyme*

Mechanical rupture of spores activates most, if not all, of the enzymes dormant in intact spores (*4*). Heat activation prior to rupture gives greater glucose-oxidizing activity in the extracts, presumably because of the release of endogenous alanine (*5*). The activation of glucose oxidative activity in spore extracts deserves further comment. The amount of alanine required to stimulate glucose oxidation is of the order of 1/100 of the amount required for rapid germination. Experiments on the effects of repeated heat activation in the presence or absence of alanine have suggested the interesting concept of a fixed, preformed pool of potentially activatable glucose-oxidizing activity in spores (*29*). Only a fraction of this potential activity is released during one heat shock. A subsequent heat shock destroys the active (heat sensitive) enzyme present, but also activates another portion of the dormant enzyme. These results are important in that they suggest the possibility that increasing amounts of an enzyme during germination may be due only to the process of fully activating preformed enzyme. A recent report has suggested that the heat activation is only apparent, being due to heat inactivation of DPNH oxidase (*44*). Whether this explanation can fully satisfy the data remains to be seen.

Convincing evidence has been obtained that at least some enzymes in spores are bound to macromolecular structures, thus conferring heat resistance. These enzymes become heat labile if released from their bound form during the normal process of germination or by sonic disruption of the particle-bound enzyme. Thus in vegetative cells alanine racemase is heat labile, whereas in spores it is normally heat stable (*45*). However, if spores are ruptured by sonic oscillation, a soluble enzyme

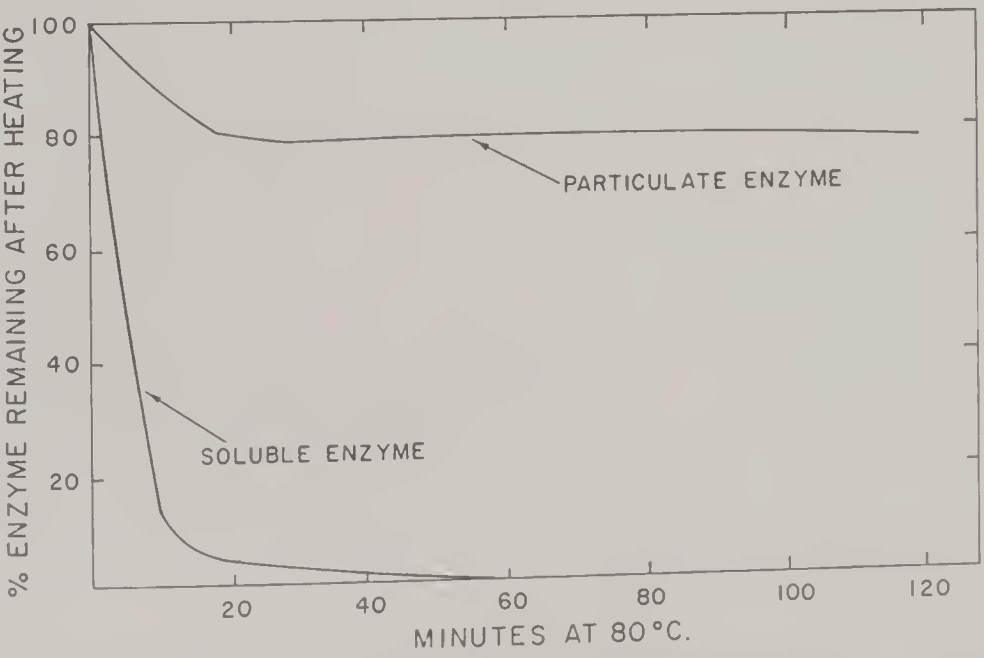

FIG. 7. Heat inactivation of soluble and particulate alanine racemase of spores. From Stewart and Halvorson (*45*).

fraction is released and is found to be heat sensitive. The particulate enzyme fraction also present (40,000 g) is heat resistant (see Fig. 7).

Further sonic treatment of the particulate fraction again produces both a soluble heat-sensitive fraction and a particulate resistant fraction. Spore catalase and adenosine ribosidase exhibit similar sensitive and resistant fractions. The nature of the colloidal particle binding these enzymes is unknown. In this connection, it has been demonstrated that heat stability may be conferred reversibly on yeast catalase by its absorption onto lipid or RNA [for a discussion of this, see p. 104 in (*11*)]. As we have seen, large amounts of DPA and a special peptide are present in spores and are released during germination. A number of workers have speculated on the possibility that the thermostability of spore enzymes may be mediated by a stabilizing structure formed, in part, by these special materials characteristic of spores, such as a DPA-calcium-protein complex. That there may be a nonspecific mechanism in spores for protecting enzymes is indicated by investigations on a penicillin-resistant strain of *Bacillus cereus* [Harel and Halvorson, unpublished results referred to in (*4*)]. The penicillinase which had been developed in this strain was passively transferred in the heat-resistant intact spores. Thus protection was conferred on a "foreign" protein not involved in the normal metabolism of these cells.

The data obtained on alanine racemase and similar enzymes again indicate the existence of significant amounts of preformed (bound) enzyme which is released on germination. In these instances, the bound protein is enzymatically active, whereas in the glucose-oxidizing system the data suggest that the heat-stable system is not enzymatically active. Perhaps in the latter case release of the bound enzyme frees active centers which may then combine with their substrate.

3. *The Role of Enzyme Induction*

In the following discussion, we shall define enzyme induction as an increase in the rate of synthesis of a specific protein. This mechanism shall be distinguished from activation, which shall be defined as any other process not involving enhanced synthesis yet resulting in increased enzyme activity. The available data give us examples of the activation, both *in vivo* and *in vitro*, of existing enzyme systems by the addition of substrates which were probably largely depleted during sporulation. Enough is now known about the enzyme content of spores to suggest that they may contain most of the enzymes present in vegetative cells. In this case, altered enzyme activities are due to quantitative differences, which may or may not be equivalent to actual increases in the rate of synthesis or concentration of the enzyme protein. Since enzyme activation phenomena do play an important role in germination, the question remaining is whether enzyme induction is also involved.

Germination is associated with a burst of oxidative activity. The attractive suggestion has been made [see discussion (*11*), p. 118] that cofactors required for these reactions may be present in the spore predominantly as DPNH, TPNH, and ATP. The addition of small amounts of reducible substrates might initiate the rapid oxidation of the pyridine nucleotides and the hydrolysis of ATP. Thus hydrogen and phosphate acceptors would become available for extensive endogenous respiratory activity. It was further pointed out that the activation of *Neurospora* spores is accompanied by a precipitous decline in ATP and rise in ADP (adenosine diphosphate) concentration. A concomitant release of dipicolinic acid during germination would of course further stimulate oxidative activity in serving as an electron acceptor. To what extent catalytic levels of endogenous substrates and a dearth of oxidized coenzymes can indirectly be responsible for the enhanced enzymatic activities associated with germination remains to be seen. Data on the relative concentrations of these substances immediately before and after germination would be very useful.

As mentioned previously, sporulation in aerobic bacilli is also immediately preceded by a burst of oxidative activity. This critical "presporulation" period is associated with the enhanced activity of DPNH oxidase (*46*), glucose dehydrogenase (*44*), heat-stable catalase (*47*), systems oxidizing acetate and poly-β-hydroxybutyric acid, and enzymes involved in DPA synthesis. We have seen that a major factor in the initiation of sporulation is the exhaustion of an exogenous energy source. Spore formation may thus be stimulated by removal of a repressor of the above enzyme systems (*48*). In fact, excess glucose inhibits DNA synthesis as well as the formation of prespores (*49*).

a. Inhibitors of RNA and Protein Synthesis. It is probable that enzyme induction is involved following germination and during the presporulation period just mentioned. Here extensive alterations in enzyme composition occur, and future sporulation of the cells is inhibited by amino acid and pyrimidine analogs (*35, 50*). However, the *immediate* germination and sporulation process (following "commitment") appear to be quite refractory to inhibitors of RNA and protein synthesis. Recent data demonstrate that no net RNA synthesis occurs during sporulation, in spite of an active RNA turnover (*51*). Under such circumstances, induced enzyme synthesis is possible (*52*), just as induced enzyme synthesis is possible in the absence of net protein synthesis if protein turnover occurs (*53*). However, the addition of 8-azaguanine and 2,6-diaminopurine following commitment have no inhibitory effect on sporulation (*50*), and 8-azaguanine should inhibit induced enzyme synthesis (*54*).

It has been demonstrated that the induction of maltozymase in yeast (*55*) and penicillinase in *Bacillus cereus* (*56*) is very sensitive to ultraviolet (UV) irradiation. Particularly in the former case, an intimate relationship was demonstrated between RNA and protein synthesis. Studies have also been made on the effect of irradiation on spores prior to germination [e.g., see (*57*)]. Doses sufficient to destroy the capacity for cell division did not interfere with germination in glucose. Glucose is also a known inhibitor of enzyme induction (*48*).

To summarize, the available evidence suggests that enzyme induction is not essential in the immediate germination and sporulation process.

4. *Respiratory Adaptation*

In connection with speculations on the mechanism of altered enzymatic activities, it may be relevant to mention the phenomenon of "oxygen-induced enzymes." When anaerobically grown cells of yeast (*58*) or *Pasteurella pestis* (*59*) are exposed to oxygen, they acquire aerobic enzymatic activities. Tricarboxylic acid cycle enzymes and typical cytochrome bands appear within a few hours after adapting the cells to aerobic conditions. In the case of the yeast, a careful study was made of the various cytochrome bands present or absent under anaerobic compared to aerobic conditions. These and other data on the kinetics of cytochrome c formation lead to the suggestion that a conversion of anaerobic to related aerobic cytochromes occurs during respiratory adaptation. More recently, investigations in this system have shown that anaerobically grown yeast possess a lactic acid dehydrogenase incapable of reducing cytochrome c. During adaptation to aerobic metabolism this enzyme is apparently converted to L-lactic acid cytochrome c reductase (*60, 61*). Thus the new enzymatic activity capable of reducing cytochrome c may arise not by *de novo* synthesis but by the activation or transformation of pre-existent protein. The analogies between the phenomenon of "oxygen-induced enzymes" and the process of germination with respect to enhanced aerobic metabolism may of course be superficial. Nevertheless, it could prove fruitful to bear them in mind when speculating on the mechanisms underlying altered enzymatic activities during germination and sporulation.

III. Sporulation of the Cellular Slime Mold

Compared to bacteria, slime molds have arrived at an even more complicated procedure for the formation of their spores. Individual vegetative cells (myxamebas) become transformed into spores, as occurs in the spore-formnig bacteria. However, during this sporulation process, other "somatic" cells are engaged in sacrificing themselves to create a cellulose stalk on which the spore cells will rest. It will become obvious that there are many striking analogies between the events of bacterial sporulation and the more elaborate behavior of the slime molds. Very little has been done with respect to the germination of slime mold spores; we shall be primarily concerned with the multicellular differentiation process resulting in sporulation.

A. The Initiation of Multicellular Differentiation

When the individual myxamebas have exhausted their bacterial food supply, they are stimulated to aggregate, forming a new multicellular "organism" called a migrating pseudoplasmodium. The cells aggregate in response to a chemotactic hormone. Further differentiation into the fruiting body or sorocarp proceeds and is completed in the absence of exogenous nutrition (*62*). The cells in the foremost third of the pseudoplasmodium are destined to form the stalk of the eventual fruiting body. The other two-thirds of the cells become the spores. The critical nature of food shortage in stimulating this morphogenic transformation has been demonstrated by some very simple experiments. Pseudoplasmodium formation is indefinitely delayed by continually supplying fresh bacteria to the myxamebas. If, in the presence of bacteria, a pseudoplasmodium is completely broken up to yield separate myxamebas, the individuals will continue vegetative life. However, a whole or even a fraction of a partially disorganized pseudoplasmodium will develop normally in the presence of bacteria. Thus, athough an exogenous food supply can indefinitely delay morphogenesis, the achievement of a certain degree of specialization during differentiation precludes inhibition by an exogenous energy source. It is interesting that plasmodium formation of a true slime mold can be inhibited by addition of glucose to the medium (*63*). This situation is quite reminiscent of the depressing effect of glucose on bacterial sporulation discussed earlier. We are also reminded of Foster's suggestion that the primary event in the metamorphosis of a vegetative cell to a spore may be a dissociation of the dependence of endergonic reactions on an exogenous energy source. At this point, endogenous high molecular weight compounds are used for energy sources as differentiation begins.

B. The Energy Requirement of Differentiation

During bacterial sporulation, exogenous nutrients are present in the liquid medium, having either been added by the investigator or produced by the bacteria. Slime molds differentiate on a solid agar surface in the complete absence of nutrition, making it very obvious that all energy sources must be of endogenous origin.

1. *Alterations in Respiration*

The respiration of *Dictyostelium discoideum* during differentiation on washed agar is shown in Fig. 8 (*64*). Oxygen uptake per cell (open circles) is highest at the ameba stage, then drops sharply and declines linearly with time from the migrating pseudoplasmodium to the final sorocarp stage. The first drop probably reflects a shift from the utilization of an initially large free amino acid pool (and other small molecules) to the less readily available endogenous protein. In fact, during the transition from the amebas to the migrating pseudoplasmodium there is a drop in the size of the free amino acid pool to 30% of its original value; thereafter it remains fairly constant. The migrating pseudoplasmodium stage also marks the beginning of the major period of endogenous protein utilization. It is reasonable to expect the starving cells

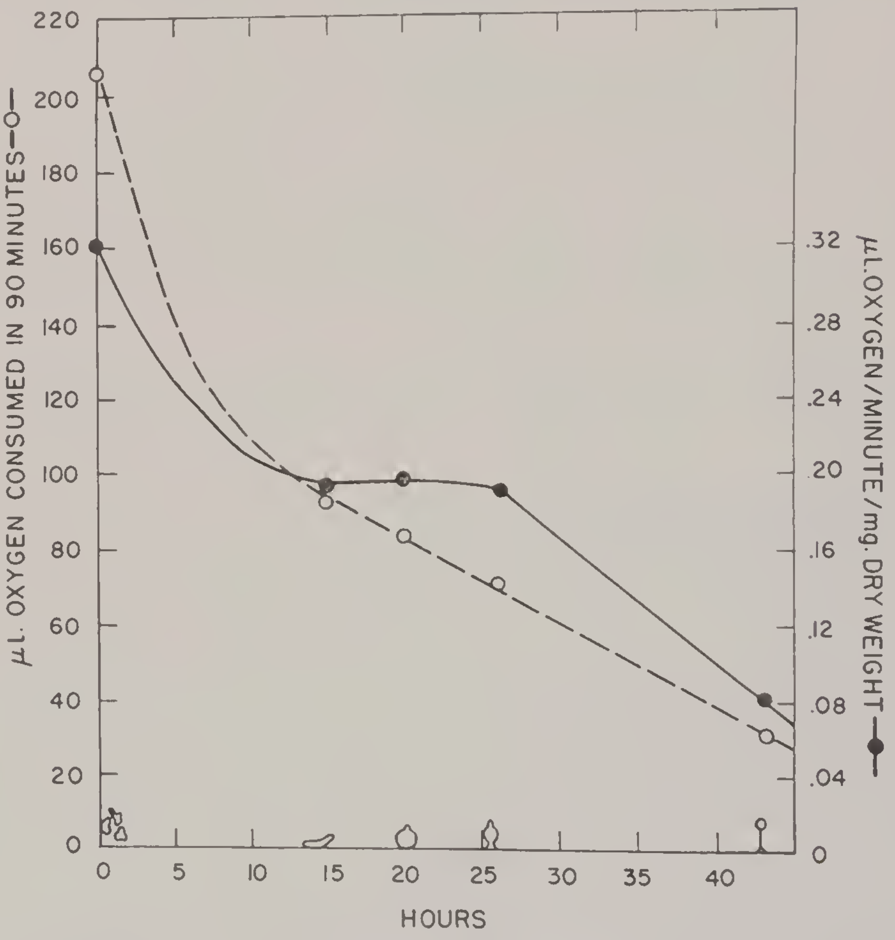

FIG. 8. Respiration of *Dictyostelium discoideum* during multicellular differentiation. From Liddel and Wright (*64*).

to consume an ever-decreasing amount of oxygen per unit weight as their supply of endogenous reserves decreases during differentiation.

Respiration based on dry weight (Q_{O_2}) (filled circles) exhibits an interesting plateau between the pseudoplasmodium and culmination stages, before a final drop at the sorocarp stage. Since glucose can stimulate respiration during this plateau period, substrates rather then enzymes are apparently limiting respiration (see Table III). The downward break in the Q_{O_2} curve after the culmination stage may indicate a critical depletion of endogenous oxidizable substrates. The sorocarp stage is the period during which the addition of exogenous glucose to the cells in a salt solution provides the greatest stimulation to the respiration, raising the level to that of the plateau region (Table III). This finding suggests that the decrease in respiration at the fruit stage during normal development is due to substrate depletion rather than to any deficiency in the many enzymes and coenzymes that participate in the activation and oxidation of glucose. It is clear that depletion of a bacterial food supply initiates the differentiation process, and progressive depletion of endogenous energy sources may in part be responsible for the cessation of differentiation.

TABLE III

O_{O_2} AT FOUR STAGES OF DEVELOPMENT[a]

Stage	Q_{O_2} [b]	S.D.[c]	Number of experiments	Avg. % stimulation by glucose	S.D. (%)	Number of experiments
Ameba	0.271	0.011	9	0	0	11
Migrating pseudoplasmodium	0.199	0.021	17	0	0	7
Preculmination	0.214	0.019	10	9.4	4.1	5
Young sorocarp	0.147	0.030	9	40	13.7	13

[a] From Liddel and Wright (*64*).

[b] Microliters O_2 uptake per minute per milligram dry weight.

[c] Standard deviation.

2. *Utilization of Endogenous Reserves*

The over-all metabolism of the differentiating slime mold involves the conversion of endogenous protein to carbohydrate (*65, 66*). Employing S^{35}-methionine-labeled cells, it could be shown that the amino acid pool drops to about 30% of its initial value, the ethanol-soluble protein to 60%, and the ethanol-insoluble protein to about 80% of its initial value over the course of differentiation (*67*) (see Fig. 9). It is not surprising

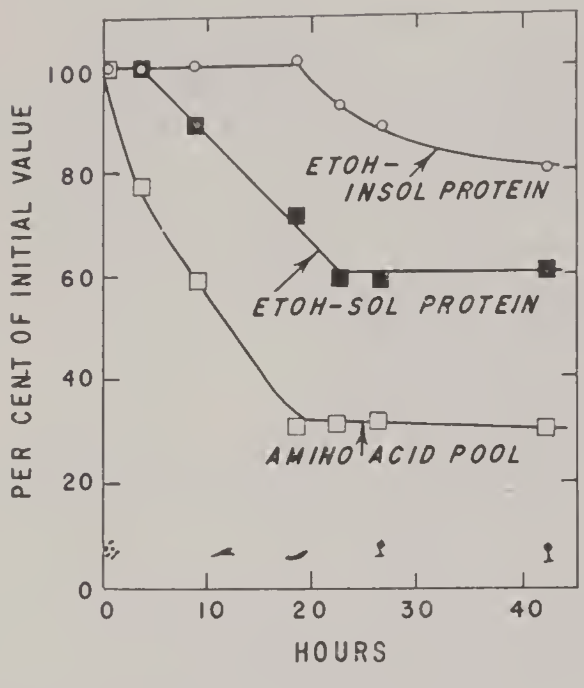

FIG. 9. Utilization of endogenous amino acids and protein during slime mold differentiation. From Wright and Anderson (*67*).

that an early utilization of the amino acid pool would occur in slime mold differentiation. A similar depletion is seen durng bacterial sporulation, as mentioned earlier, and the pool amino acids are incorporated into protein during nitrogen starvation in both yeast (*68*) and *Escherichia coli* (*69*). Another comparison can be mentioned in reference to the early utilization of ethanol-soluble protein by the slime molds. In starving *E. coli* cells, at least a fraction of the ethanol-soluble protein is degraded to amino acids which are incorporated into the residual, ethanol-insoluble protein. The soluble protein is "expendable" in that its use does not impair cell viability. The cessation of major protein utilization at culmination may reflect the fact that protein depletion largely occurs in the presumptive stalk cells (*65*), which have now reached the end of their metabolism.

Recent studies have shown that losses during differentiation in dry weight, protein, and RNA content are not correlated with morphological events (*70*). The rate of loss of these cellular constituents is the same in wild type as in various mutants unable to aggregate and/or fruit. Apparently the depletion of endogenous reserves is rather a function of the time the cells remain on agar in the absence of an exogenous food supply. The utilization of these materials is thus a necessary, but not a sufficient, condition of morphogenesis.

A correlation has been found between polysaccharide accumulation and morphological events. Total reducing power has been shown to increase during development (*66*), and Fig. 10 demonstrates that the

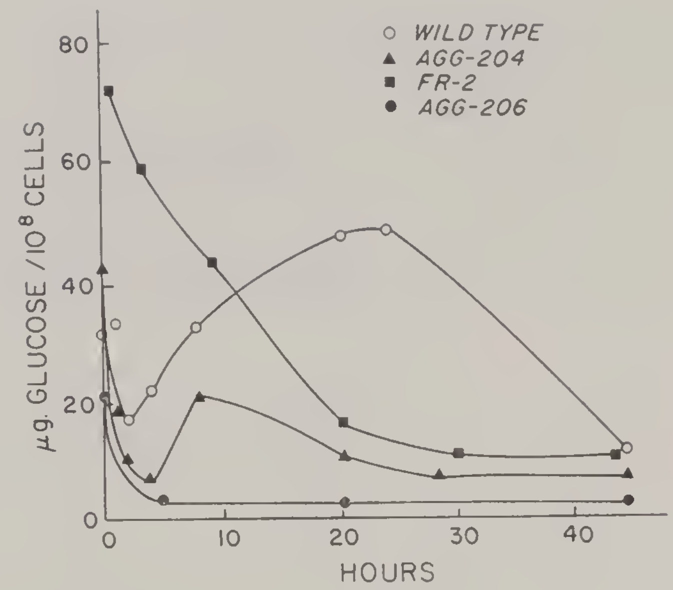

FIG. 10. The correlation of TCA-soluble polysaccharide synthesis and normal development in *Dictyostelium discoideum* (*70*).

synthesis of a bound glucose fraction is linked to normal morphological development (*70*). The ordinate represents the difference in glucose content of hydrolyzed and unhydrolyzed samples. It can be seen that the morphological mutants have failed to link their endogenous catabolism to the biosynthesis of this polysaccharide. Presumably in the normal course of differentiation endogenous protein is degraded to amino acids which are in turn deaminated, becoming tricarboxylic acid cycle intermediates and eventually glucose and cellulose. The presence of a number of enzymes involved in such reactions have been demonstrated *in vitro* (*71*). A reversal of the tricarboxylic acid cycle is also considered to be responsible for the differentiation of the resistant sporangium of the water mold *Blastocladiella emersonii* (*72*).

a. Permeability Considerations. One might speculate that the nature and extent of the utilization of endogenous reserves during development could vary enormously, depending upon the availability of exogenous energy sources. That this is not the case early in slime mold development has been shown for one amino acid and for glucose. The data in Table IV demonstrate that the cells are almost impermeable to methionine and glucose in the early stages of differentiation. After exposure of the cells to S^{35}-methionine or C^{14}-glucose at three stages of development, these compounds were isolated from the cells and their specific radioactivity was determined (*38, 73*). Clearly the myxamebas are very poorly permeable to these substances, a fact that may account for their lack of response to glucose (Table III). In the case of methionine, it was shown that even when exogenous methionine was maximally "permeable" (at the pseudoplasmodium and preculmination stages), it did not

TABLE IV
SPECIFIC RADIOACTIVITY OF INTRACELLULAR COMPARED TO EXTRACELLULAR S^{35}-METHIONINE AND C^{14}-GLUCOSE[a]

Methionine source	Exogenous methionine (%)	Glucose source	Exogenous glucose (%)
Exogenous	100	Exogenous	100
Ameba	8.3	Ameba	1.4
Pseudoplasmodium	66.4	Preculmination	52.6
Sorocarp	28.2	Sorocarp	32.3

[a] Data from Wright and Anderson (*38*) and from Wright and Bloom (*73*).

"expand" the endogenous pool of methionine. That is, as Table V indicates, the size of the endogenous methionine pool was a function only of the stage of development, not of the concentration of exogenous methionine. However, it is clear that the specific radioactivity or *exchangeability* of endogenous methionine is affected by *both* the stage of differentiation and the concentration of exogenous methionine. The point to be made at this time is that, regardless of the environment, the differentiating slime mold must rely exclusively on an endogenous source of methionine. Certain other amino acids, as well as glucose, can expand their intracellular pools, particularly at the later stages of development (*64, 74, 75*). However, if differentiation were dependent on protein turnover, the rate of methionine formation from endogenous sources—probably protein degradation—could in effect be a limiting factor controlling development, regardless of a nutritionally rich or poor environment. Data on the endogenous, as well as the glucose-stimulated,

TABLE V
EXCHANGEABILITY OF EXOGENOUS WITH ENDOGENOUS METHIONINE[a]

Stage	Exogenous S^{35}-methionine (μmoles/ml.)	Micromoles methionine per cell aliquot	C.p.m./μmole endogenous methionine $\times 10^6$
Ameba	0.04	0.099	0.014
Ameba	0.40	0.105	0.133
Pseudoplasmodium	0.04	0.018	0.120
Pseudoplasmodium	0.40	0.017	1.061
Sorocarp	0.04	0.021	0.040
Sorocarp	0.40	0.020	0.44

[a] From Wright and Anderson (*38*).

respiration of sorocarps harvested from a rich medium, as compared to washed agar, suggest that endogenous substrate depletion occurs as readily in both cases (*64*).

There appears to be a general dependence of differentiating systems, at least in the early stages, upon endogenous reserve materials as a source of energy. It is therefore of particular interest that permeability restrictions exist in the slime molds that necessitate such a dependence upon an intracellular source of certain metabolites.

b. Protein Synthesis. As noted earlier, the rate of protein degradation is not constant, but appears to reach a peak at the preculmination and culmination stages of differentiation. On the other hand, protein synthesis, as judged from the incorporation of S^{35}-methionine into protein, is fairly constant during development (*38*). Initially the data suggested that the rate of protein synthesis was seven or eight times greater at preculmination than at the ameba stage. For example, in the second column of Table VI, it can be seen that the increase in specific radioactivity of protein isolated at the pseudoplasmodium stage was six times that of the protein isolated at the ameba stage (following comparable exposures to exogenous S^{35}-methionine at each stage). However, as we have seen (first column), the labeling of endogenous methionine is much more extensive at the pseudoplasmodium as compared to the ameba stage of development. Thus calculated rates of incorporation of pool methionine into protein give comparable values in the two instances. Because of the impossibility of completely labeling endogenous methionine, and because of a very complex relationship between the "free" methionine pool and its incorporation into protein, it is difficult to give a reliable value for the replacement of protein by pool methionine (*38*). It is approximately 7% per hour at preculmination.

TABLE VI

THE RATE OF PROTEIN SYNTHESIS AT TWO STAGES OF DEVELOPMENT[a]

Stage	C.p.m./μmole endogenous methionine $\times 10^6$	C.p.m./mg. protein increase per 30 min.	Incorporation (%)
Ameba	0.13	840	7.9
Pseudoplasmodium	1.06	5,040	6.2

[a] From Wright and Anderson (*38*).

3. *The "Limiting Step" in Morphogenesis*

The observation was made that exogenous histidine stimulates morphogenesis in *D. discoideum* (*74*). Experiments designed to confirm and extend these results revealed that comparable stimulation could be achieved by the addition of glucose, phosphate, magnesium, or certain other amino acids (*76*). Table VII shows the effect of a few substances on sorocarp formation. Additions were made to washed agar buffered at pH 6.2, and the number of fruits was determined after incubation for 20 hours at 22°. The inactivity of acetate and salts such as sodium sulfate indicate that the positive effects probably are not due to the ionic strength or buffering capacity of the stimulants. The mechanisms by which these compounds exert their effect on the rate of differentiation is not yet known. A point of interest at present concerning these results is the dissimilarity of the stimulants. Since they must have different mechanisms of action, at least initially, a number of mechanisms for stimulating morphogenesis are indicated. In another sense, the data suggest a very central limiting step susceptible to influence in a number of ways. A limitation in energy sources might be considered a likely candidate. Phosphate stimulation could be due to enhanced high energy phosphorylation; magnesium could act by stimulating proteolytic enzymes, etc. In this connection, 2,4-dinitrophenol stimulates respiration about twofold in *D. discoideum* (*64*), thus suggesting a limitation of phosphate acceptors.

TABLE VII

EXOGENOUS STIMULANTS OF DIFFERENTIATION (*76*)

Additions	Relative fruits (%)
Histidine 0.04 M	100
None	10
PO_4(Na) 0.01 M	133
PO_4(Na) 0.004 M	41
PO_4(Na) 0.004 M + histidine 0.04 M	154
Acetate (Na) 0.003 M	9
Acetate (Na) 0.02 M	18
Glucose 0.05 M	92
Glucose 0.05 M + histidine 0.04 M	250
Na_2SO_4	11

C. MECHANISMS OF ALTERED ENZYME ACTIVITIES DURING DIFFERENTIATION

1. *In Vivo Evidence*

It is quite clear from watching a slime mold make a cellulose stalk that striking changes in the activity of metabolic pathways responsible for cellulose synthesis occur at culmination. It is also obvious, from the respiration curve in Fig. 8, that less oxidative activity occurs in spores than in amebas. Evidence already discussed indicates that the explanation for the low respiratory activity of spores lies in a deficiency of substrates, not of enzymes.

Additional more specific evidence for shifts in metabolic pathways during development has been obtained from studies on the metabolism of 6-C^{14}- and 1-C^{14}-labeled glucose (73). The ratio of the rate of $C^{14}O_2$ evolved from the 6 to the 1-C^{14}-glucose was determined using intact cells of *D. discoideum.* In the slime mold the C-6/C-1 ratio is a function of (*a*) the relative activity of the Embden-Meyerhof (EM) and hexose monophosphate shunt pathways, and (*b*) the extent to which the triose isomerase reaction is limiting, depending upon the intensity of the EM pathway. Both (*a*) and (*b*) are affected by the concentration of exogenous glucose, which therefore influences the C-6/C-1 ratio at any stage of differentiation. However, both the quantitative and qualitative effects of exogenous glucose on the ratio are functions also of the stage of differentiation. This dependence can be attributed to endogenous changes in the molarity of glucose, as well as to a shift during development to a gluconeogenic metabolism in which protein is used as an energy source. Since it is possible to influence the pathways of glucose catabolism at exogenous concentrations comparable to those present *in vivo* at preculmination, it appears that the changes in intracellular glucose molarity that normally occur during morphogenesis are such as to effect the pathways of hexose metabolism (77).

2. *In Vitro Evidence*

A number of enzymes involved in oxidative metabolism were found to have maximal specific activities *in vitro* (based on protein or dry weight) at preculmination (73). Histochemical studies demonstrated nonspecific dehydrogenases to be maximally active also at this stage (78). Subsequently it was found that some of these alterations in specific enzyme activity *in vitro* do not reflect *in vivo* changes in the concentrations of the relevant proteins (79). Rapid *in vitro* inactivation of some enzymes, but not others, occurs only in extracts prepared at early stages of differentiation, not in extracts prepared at preculmination. This specific enzyme inactivation was found to be due to a heat-labile factor present only in extracts prepared in the early stages of differentiation. The destructive action of the presumed enzyme responsible for the inactivation of isocitric dehydrogenase, for example, can be prevented by the presence of isocitrate. This is shown in Table VIII. At each of the three indicated stages of development, comparable aliquots of cells were harvested in the absence and presence of isocitrate. Extracts were prepared, and isocitric dehydrogenase activity was determined for each sample immediately and again after a 2.0-hour incubation period at 21°. It can be seen that isocitrate prevented enzyme inactivation at early aggregation. There is suggestive evidence in other developing systems [germinating seeds (*80*) and mouse embryos (*81*)] for differential enzyme inactivation *in vitro* as a function of the stage of differentiation.

TABLE VIII

SPECIFIC ACTIVITIES[a] OF ISOCITRIC DEHYDROGENASE[b]

		Stage of differentiation		
Isocitrate	Hours preincubated	Early aggregation	Preculmination	Young fruit
Absent	0	2.83	3.72	2.21
	2.0	0.13	2.77	1.53
Present	0	3.84	3.86	3.12
	2.0	3.08	3.07	3.03

[a] Δ 340 mμ per milligram protein per min.

[b] From Wright (*79*).

This phenomenon is apparently only an artifact of extracts and has no known significance with respect to biochemical mechanisms of differentiation *in vivo*. It does serve to emphasize the completely different chemical and physical environment of enzymes in extracts compared to enzymes in intact cells and also to emphasize the dangers of extrapolating from results obtained *in vitro* to conditions *in vivo*.

Of the specific enzymes which have been examined thus far, all are present at each stage of development. Two which are stable in extracts do not change significantly during development. Specific activities are seen in Table IX. Enzymes such as these should be of particular importance to an over-all metabolism in which amino acids are converted

TABLE IX

SPECIFIC ACTIVITIES[a] OF GLUTAMIC DEHYDROGENASE AND ALANINE-α-KETOGLUTARIC ACID TRANSAMINASE[b]

Stage of differentiation	Dehydrogenase	Transaminase
Early aggregation	50.0	5.50
Preculmination	50.7	4.72
Young fruit	33.0	5.52

[a] Δ 340 mμ per milligram protein per min.

[b] From Wright (*79*).

to carbohydrate. Although the enzyme concentration does not change (Table IX), the rate of glutamate oxidation *in vivo* has been shown to increase sixfold over the course of differentiation. Glutamate accumulates in the cells during development. Knowing the K_M of glutamic acid dehydrogenase, it was shown that alterations in the $(S)/K_M$ ratio during differentiation could fully account for the enhanced enzyme activity *in vivo* (*71*).

3. *Sequential Substrate Availability*

The lack of change in the rate of protein synthesis and in the concentration of particular enzymes suggested speculation on the possible importance of altered endogenous substrate concentration as a controlling factor in biochemical differentiation. It is actually possible, in a system of constant enzymatic constitution, that sequential substrate utilization could account for major metabolic changes observed during development. It is known that in many tissues the concentration of endogenous substrate is much lower than will saturate the enzyme; i.e., the reaction is substrate limited (*82*). That this is also true in starving slime mold cells is very probable, particularly in view of the data presented earlier indicating a progressive endogenous substrate depletion as development progresses. Theoretically, beginning with a limiting amount of endogenous protein, one can imagine the sequential formation of various intermediates, eventually resulting in the synthesis of cellulose (*79*). Needless to say, life is more complicated than this. The model merely serves to illustrate the possible contributing role of sequential substrate availability in the mechanisms of biochemical differentiation.

IV. Seed Germination

A. THE ENERGY REQUIREMENT OF GERMINATION

1. *The Initiation of Germination and Respiration*

In contrast to the situation in bacteria and slime molds, the presence or absence of an exogenous nutrient is not particularly critical in the initiation of plant development. It is the moisture content of a seed which in large part determines the rate of its respiration and germination. The importance of hydration in bacterial spore germination is more difficult to assess, owing to the aqueous environment in this case (*8*).

If barley seeds are exposed to water at any season, germination begins and within 24 hours there occurs a measurable breakdown of reserves and synthesis of polysaccharides. The embryo is the center of this initial activity; endosperm metabolism is still in a lag phase, as we shall see below. Other stimulants of seed germination include manganese sulfate (*83*), thiourea (*84*), and lactate (*85*).

A sequence of respiratory stages are associated with the process of germination. During the first 20 hours, germinating barley seeds exhibit the pattern of oxygen consumption (open circles) and CO_2 evolution (filled circles) shown in Fig. 11. From data such as these, respiratory quotients have been obtained and correlated with the sequential utilization of reserve materials (see below).

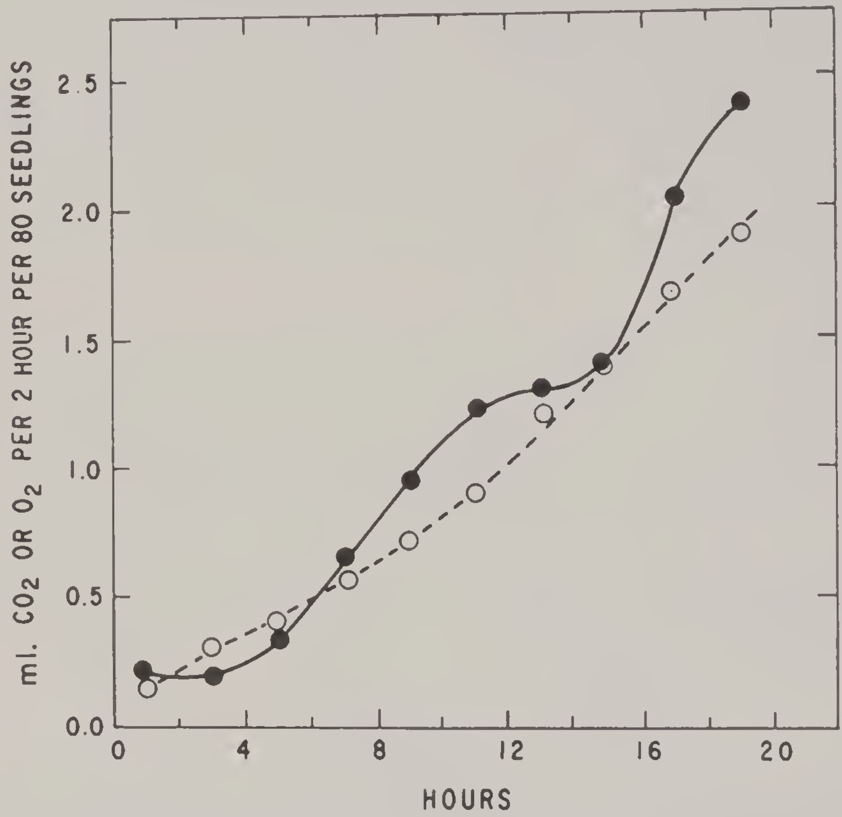

FIG. 11. Oxygen consumption and CO_2 evolution in germinating barley seeds. From James and James (*86*).

a. Electron Transport. Cytochrome oxidase is apparently active only in plant embryos and during the initial stages of development in grasses (*87*). The presence of cytochrome oxidase was demonstrated by the use of specific substrates and inhibitors, and by spectroscopic analysis. Later in development a CN⁻- and CO-insensitive system replaces cytochrome oxidase. Flavoprotein enzymes are present, and flavin content as well as peroxidase activity increases as the plant ripens. Polyphenoloxidase may be the terminal oxidase in certain higher plant tissues.

2. *Utilization of Endogenous Reserves*

The reserve food supply of seeds usually consists predominantly of carbohydrates or fats. In many seeds proteins also serve as an important fraction of the storage materials. A great variety of hydrolytic enzymes are present which degrade proteins, carbohydrates, and fats. Gradients of proteinases and peptidases are found in seeds, and proteolytic, amyloclastic, and lipolytic activities increase during germination.

The nature of the reserve materials in a seed and the sequence in which they are used in part determines the ratio of the volume of CO_2 evolved to the volume of O_2 during germination. This respiratory quotient (R.Q.) is theoretically 1.0 for the complete oxidation of carbohydrate, and the value is 0.7 for fats. For example the R.Q. was determined on the 4th day of germination for a number of fatty seeds (castor bean, peanut, cotton, sunflower, flax, and hemp), two seeds rich in protein (Windsor bean and pea), and two starchy seeds (maize and wheat). The R.Q. for the seeds rich in protein and fat was about 0.8, and for the starchy seeds was 1.0.

Alterations in the respiration of germinating barley seeds have been presented in Fig. 11. The seeds were kept in the dark to ensure their complete dependence on endogenous reserves. During the first 24 hours, starch decomposition in the endosperm was too small to be measured, but it rose to 340 mg. per seed during the second 24-hour period. Initially the embryo develops at the expense of its own reserves; the redistribution of some of these materials during the first and second 24-hour period is shown in Table X (*86*).

TABLE X
Distribution of Endogenous Materials in Barley Seeds[a]

	Hexose (mg. per 100 embryos)	
Materials	First day	Second day
Formed		
Cellulose	1.1	1.8
Hemicellulose	3.1	13.1
Maltose	4.4	4.4
	8.6	19.3
Broken down		
Raffinose	5.9	0.0
Sucrose	10.0	5.3
Fats[b]	(2.8)	2.8
	18.7	8.1

[a] From James and James (*86*).

[b] One milligram of fat is assumed to be equivalent to 2 mg. of hexose.

During the first day, the degradation of raffinose, sucrose, and fats can account for the observed polysaccharide synthesized, with 10.1 mg. remaining for respiration and other syntheses. On the second day, however, these degradations cannot account for the polysaccharide formed. Moreover, the respiratory consumption has risen significantly. These new demands are met by the utilization of endospermic reserves.

Various chemical analyses, coupled with R.Q. data, allow a rather convincing correlation of the over-all "respiration drift" with the sequential accumulation and subsequent exhaustion of endogenous embryonic and endospermic materials. A summary of events is shown schematically in Fig. 12, in which respiratory intensity (ordinate) is plotted against progressive stages of development. The arrows indicate the approximate stage at which the substances named are exhausted. In the first phase, a rapid development of the embryo occurs at the expense of sucrose and raffinose stored in the embryo. In phase 2, embryonic reserves are exhausted and endospermic starch is converted to dextrins which in turn form sucrose. Dextrins and sucrose reach maximal concentrations when respiration is at its peak. Sucrose is considered to be the immediate substrate of respiration in the embryo; when added exogenously, this substance stimulates respiration about threefold throughout the germination process. In phase 3, the starch has disappeared from the endosperm and respiration begins to decline. The endosperm shows a falling concentration of dextrins, and in the embryo, sucrose is diminishing for the second time.

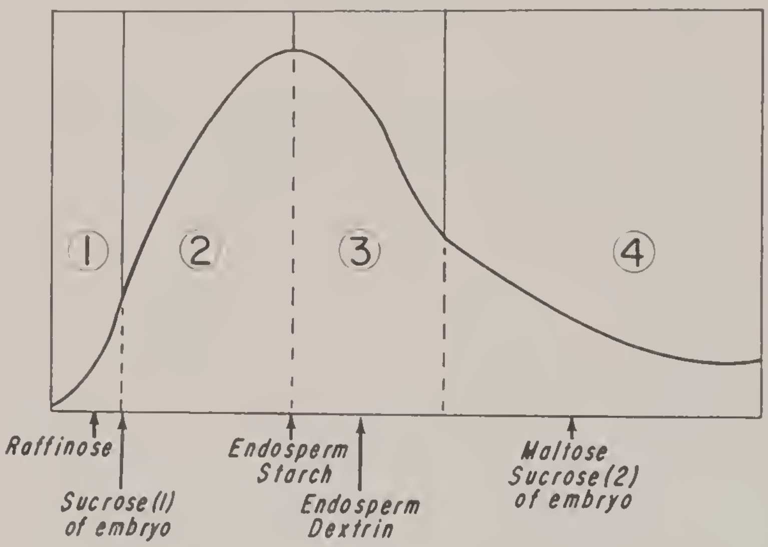

FIG. 12. The "respiration drift" during barley seed development. KEY: (1) respiration of embryonic reserves; (2) mobilization of endospermic carbohydrate; (3) exhaustion of endospermic carbohydrate; (4) mixed (including protein) respiration. From James and James (86).

TABLE XI

CHEMICAL COMPOSITION OF GERMINATING CASTOR BEANS[a]

Stage	Length of hypocotyl (mm.)	Ether extract (%)	Total reducing sugar (%)	Glucose (%)	Crude fiber (%)	N × 6.25 (%)	Ash (%)	Total (%)
0	Ungerminated	67.85	1.18	—	1.87	25.72	2.61	99.01
1	5–10	63.74	1.62	—	2.62	26.32	2.63	96.93
2	10–20	61.55	6.02	—	3.23	24.26	2.57	97.64
3	20–35	57.40	6.88	—	2.37	25.86	2.48	98.62
4	35–45	45.30	15.68	—	4.02	26.51	2.77	93.33
5	45–60	38.03	17.26	—	7.01	27.48	2.43	91.69
6	60–80	32.58	25.84	4.47	4.08	26.84	2.86	92.19
7	80–100	25.28	26.63	6.32	5.01	27.26	3.04	87.21
8	Above 100	—	—	—	—	—	—	—

[a] From Pierce *et al.* (*88*).

Table XI summarizes the redistribution of reserve materials in the germinating castor bean (*88*). As germination progresses, a striking decrease in fat content occurs accompanied by an increase in total reducing sugar. Alterations in the R.Q. value as a function of the stage of germination is given for the castor bean in Fig. 13. These seeds were germinated in darkness in order to ensure their complete dependence upon endogenous reserves. Clearly, as germination proceeds, the plant gains in an oxygen-rich substance (carbohydrate) and becomes poorer in a oxygen-poor substance (fat) (*89*). The enzymatic pathways by which fat is converted to carbohydrate in such seeds have recently been elucidated and shown to involve the "glyoxylate cycle" (*90*). The following steps describe the over-all conversion:

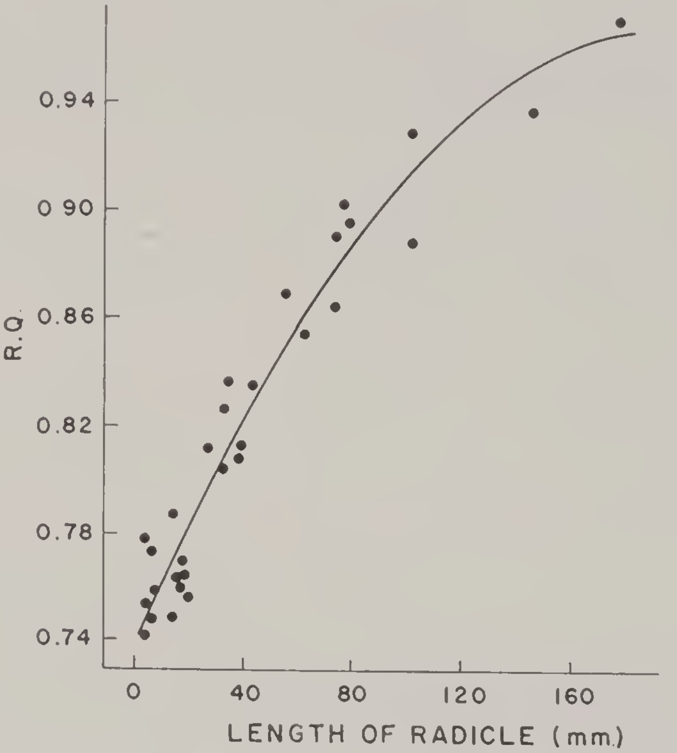

FIG. 13. Respiratory quotients during germination in the castor bean. From Daggs and Halcro-Wardlaw (*89*).

Fat → Fatty acyl CoA → Acetyl CoA → (Glyoxylate cycle) → Malate →
Phosphoenol pyruvate → (Reversed glycolysis) → Carbohydrate

An example of endogenous protein utilization during germination is shown in Table XII. Seedlings of *Triticum sativum* were placed in the presence or absence of a nitrogen source. Protein nitrogen and soluble nitrogen were then determined on the 5th day of germination. Presumably these exogenous nitrogen sources are converted to amino acids by the seedlings at the expense of endogenous carbohydrates (*91*). When the seedlings were maintained in NH_4NO_3 or urea, protein degradation was inhibited, a fact suggesting that normally the decreased concentration of endogenous free amino acids and ammonia may stimulate protein breakdown. The data could be explained also on the basis of enhanced protein synthesis in the presence of a source of soluble nitrogen.

TABLE XII
DIMINUTION OF PROTEIN HYDROLYSIS IN ENDOSPERM OF WHEAT SEEDLINGS (*Triticum sativum*) AS A RESULT OF SUPPLYING SEEDLINGS WITH SOLUBLE NITROGEN[a]

	N in 100 endosperms (mg.)		N in 100 embryos (mg.)	
Nutrient	Protein N	Soluble N	Protein N	Soluble N
Water	35.7	12.4	23.4	14.8
0.5% NH_4NO_3	47.3	16.7	25.8	27.4
Water	34.0	13.4	22.0	12.3
0.5% Urea	45.7	31.7	23.9	41.7

[a] From Paech (*91*).

B. MECHANISMS OF ALTERED ENZYME ACTIVITIES

In castor beans, at 30° the lipase activity increases with germination to a maximum at 3 days and then decreases. This lipase is considered to exist as a nonactive proenzyme in resting castor bean and cotton seeds. It can be activated through the processes of germination, or, in crude powder form, by treatment with weak acetic acid (*92*). The endosperm initially has a high fat content, which declines sharply on the 4th day. Enhanced activity of certain glyoxylate cycle enzymes occurs during germination, and by the 7th day the endosperm has been completely utilized. The respiratory activity increases during the first 6 days of germination. Alterations also occur in the relative respiratory activities

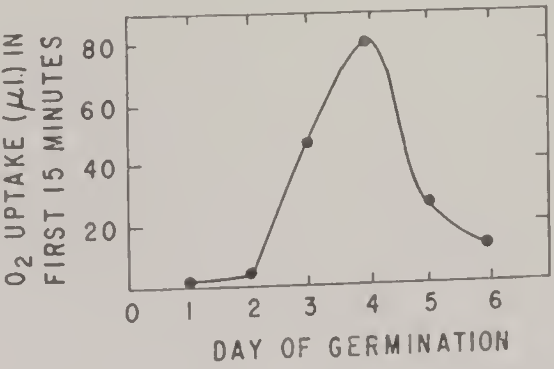

FIG. 14. Respiration of particulate preparations from developing castor beans. From Beevers and Walker (*93*).

of particulate preparations obtained from seedlings at different stages of germination (see Fig. 14). The substrate used in these studies was succinate, and in each case the sample of enzyme preparation represented five beans of the age indicated (*93*). The maximal activity was associated with a critical 4-day stage during which the major metabolic changes occur. During the first 5 days of development, dinitrophenol more than doubles the rate of oxygen uptake.

It has been demonstrated that a potent, heat-labile inactivating agent exists in 6-day extracts. These data are given in Table XIII. The active 4-day particles were resuspended in and resedimented from the 4-day supernatant (*A*), 6-day supernatant (*B*), and boiled 6-day supernatant (*C*). All manipulations were performed at 0°. The lowered activities of the older beans are attributed to the development of this inactivating agent. In view of the destructive agent described earlier in slime mold extracts, it would be of much interest to know (*a*) whether the low activities in extracts prepared on the 1st and 2nd day of germination are also due to the presence of an *in vitro* inactivating agent, and (*b*) whether substrates can influence the inactivation process. Alterations parallel to those shown in Fig. 14 occur in the Q_{O_2} values, based on nitrogen, of mitochondrial preparations obtained from seedlings of different ages. These data could be in part a reflection of the differential inactivation phenomenon discussed above. However, alterations in the activity of mitochondrial enzymes is compatible with data showing an increase in mitochondrial nitrogen up to the 5th day of development, followed by a decline (see Fig. 15).

TABLE XIII
INACTIVATION OF 4-DAY PARTICLES BY 6-DAY EXTRACTS OF CASTOR BEANS[a]

	μl. O_2 uptake in 20 min. with substrate		
Treatment	Citrate	α-Ketoglutarate	Succinate
A. 4-Day particles treated by 4-day supernatant	47	25	90
B. 4-Day particles treated by 6-day supernatant	20	4	57
C. 4-Day particles treated by boiled 6-day supernatant	39	27	105

[a] From Beevers and Walker (*93*).

Adenosine triphosphatase activity is initially fairly low in the mitochondria, but a sharp increase begins after 5 days of germination. The

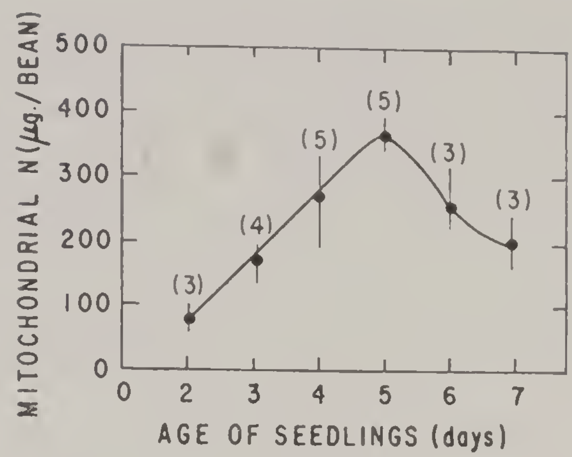

FIG. 15. Mitochondrial nitrogen as a function of seedling age. From Beevers and Walker (*93*).

activity of the supernatant, on the other hand, remains at a low level throughout (*94*). A number of the enzymes involved in the glyoxylate cycle mentioned earlier also increase in activity during germination (*95*).

It should be apparent from the above discussion that biochemical differentiation in plants appears to share a number of characteristics in common with less complex systems. Although various enzyme activities clearly change during plant development, relatively little is known at present concerning the mechanisms involved.

V. Sea Urchin Egg Development

A. THE ENERGY REQUIREMENT OF DIFFERENTIATION

1. *The Initiation of Respiration by Fertilization; Electron Transport*

In the sea urchin and amphibian it is the act of fertilization which triggers differentiation. Warburg was the first to demonstrate that fertilization brings about a fourfold increase in the oxygen uptake of the sea urchin egg (*96*). The mature egg has a relatively high respiration after its removal from the ovary. As shown in Fig. 16, the oxygen uptake then decreases until a practically constant value is reached. Upon fertilization, the level of enhanced respiration is independent of that prevailing before fertilization. The increase in oxygen uptake is less in fresh eggs than in eggs which have remained in sea water for some time before fertilization (*97*). Cell division or cleavage is completely suppressed at low oxygen tensions (*98*). Respiration does not always increase on fertilization of marine eggs; the phenomenon appears to depend on the respiratory intensity of the egg prior to fertilization (*99*).

Spectrophotometric and manometric experiments have demonstrated that the cytochrome system present in fertilized and unfertilized sea

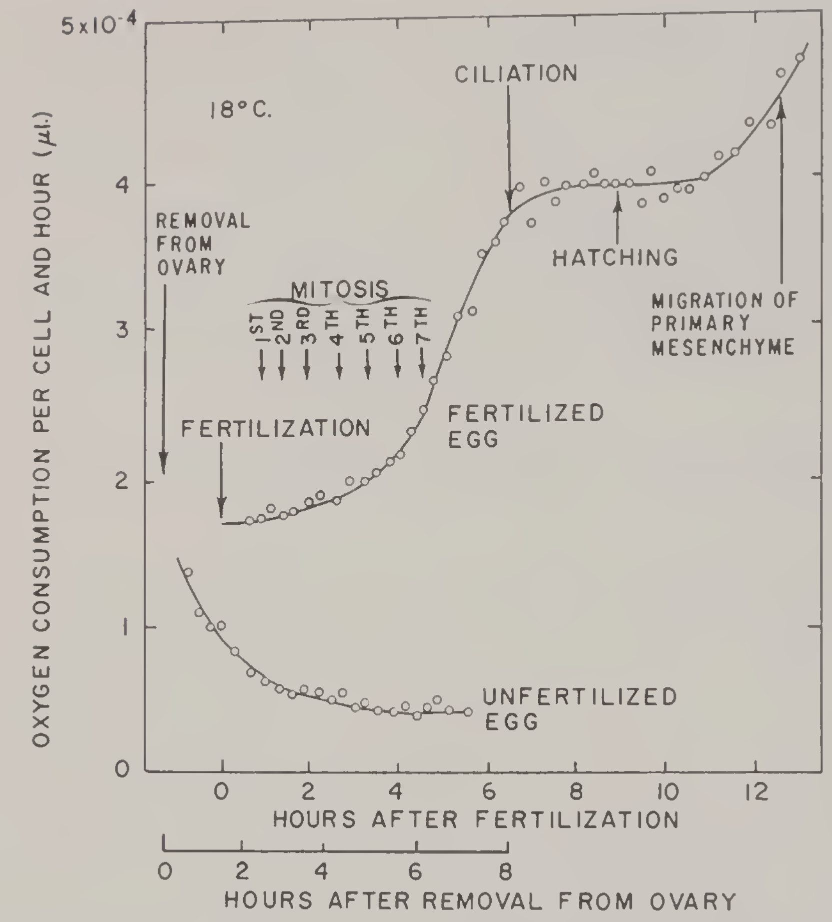

FIG. 16. Respiration before and after fertilization in the sea urchin egg. From Borei (*97*).

urchin eggs appears to be the same as that described in yeast and in mammals. Mitochondria prepared from unfertilized eggs have been shown to contain cytochromes a, a_3, b, and c. Cytochrome oxidase is also very similar to the mammalian oxidase (*100*). Participation of cytochrome oxidase in sea urchin egg metabolism is indicated from the light-reversible inhibition of both oxygen consumption and cleavage by carbon monoxide.

Oxygen consumption of the unfertilized egg is much less sensitive to HCN than is the respiration of fertilized eggs. This is shown in Fig. 17 (*101*). At a concentration of 10^{-4} *M*, however, almost complete inhibition of oxygen uptake occurs in both the fertilized and unfertilized egg. Runnström (*102*) interprets these data to mean that cytochrome oxidase functions as the terminal acceptor in both cases, but is more completely saturated with substrate after fertilization. A recent investigation on the oxidation of CO by marine eggs also gives support to this interpretation (*103*). A possible analogy can be seen in the case of yeast, in which endogenous respiration is not inhibited by CO (*104*). The addition of substrate enhances respiration, which is now strongly

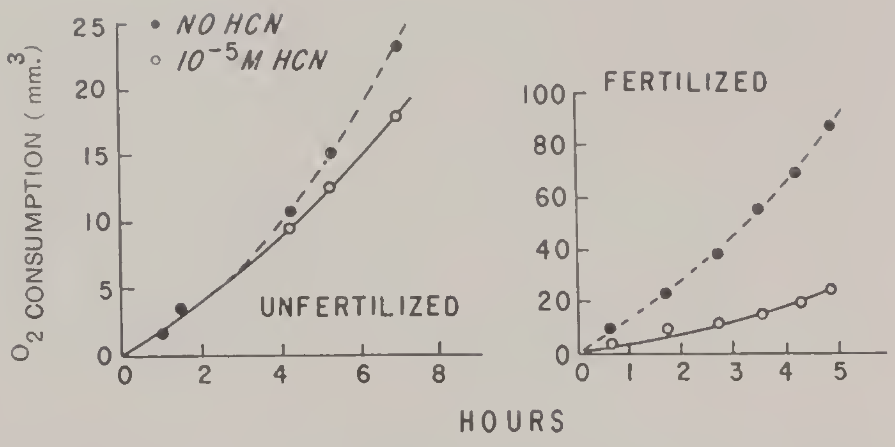

FIG. 17. Sensitivity of the respiration of fertilized and unfertilized sea urchin eggs to HCN. From Robbie (*101*).

inhibited by CO. Other indirect evidence supporting the "saturation concept" comes from experiments with dimethyl-*p*-phenylenediamine (DPPHD), which acts as an oxidizable substrate of cytochrome oxidase. The addition of $2 \times 10^{-3}\%$ DPPHD to unfertilized eggs brought the oxygen uptake to the same level as that found in fertilized eggs. Furthermore, respiration is inhibited by CO to about the same extent in fertilized and unfertilized eggs after treatment with DPPHD (*105*). One possible explanation, then, for the low level of respiration in the unfertilized egg is the existence of circumstances resulting in a deficiency of available endogenous substrates. In this connection, it is of interest that sperm extracts contain certain proteolytic enzymes (*106*) and that the activation of three proteases has been detected at the time of fertilization. On the basis of these and other results, Runnström has proposed that the spermatozoon introduces a substance which binds an inhibitor, indirectly leading to the activation of proteolytic enzymes. The addition of calcium to calcium-free homogenates of *Paracentrotus* eggs results in a marked stimulation of oxygen uptake and of proteolytic activity. The role of calcium is thought to reside in an activation of proteolytic enzymes, which are maximally active a few minutes after fertilization. Also within the first 15 minutes following fertilization acid is formed to the extent of 20 μmoles/100 mg. of nitrogen (*107*). This accumulation of acid is in part due to the appearance of new acidic groups on the egg (*108*), and may in part be a reflection of polysaccharide breakdown which occurs during this very early period of development. There is therefore some evidence favoring the view that mobilization of endogenous substrates may be an important primary event associated with the fertilization and subsequent enhancement of respiration of the egg.

Other experiments support the view that respiration prior to fertilization, and during early development, may be limited by the rate of phosphorylation. A series of substituted phenols have been shown to stimulate respiration and inhibit cleavage (*109*). The addition of dinitrocresol to the fertilized egg before or during early cleavage raises the respiratory rate to the maximum attained during the first phase of development. Data such as these suggest that the enhanced respiration following fertilization may result from an increased drain on ATP by the energy-requiring processes of the cell, thus enhancing the rate of phosphorylation and hence the rate of oxidation (*110*). In fact, the ATP and UTP (uridine triphosphate) levels drop significantly during the period of exponential increase of respiration (see Fig. 18). At the same time the monophosphates AMP and UMP increase in concentration, perhaps serving as a needed source of phosphate acceptors. A recent study on the release of respiratory control by 2,4-dinitrophenol has again emphasized the important role which may be played by phosphate acceptors produced as a consequence of the new burst of synthetic activities in the differentiating cell (*111*).

2. *Utilization of Endogenous Reserves*

Relative to the unfertilized egg, significant decreases have been found to occur in the nitrogen, carbohydrate, and lipid content of eggs during early development (*112, 113*). A significant decrease in glycogen occurs within 10 minutes after fertilization (*114*), and ammonia is liberated during this period (*115*). At the gastrulation stage of development, two soluble protein antigens had dropped to a value of less than 20% of that found in the unfertilized egg (*116*). The appearance of new antigens during development has also been described (*117*). However, alterations in protein patterns during development do not necessarily result from changes in the rate of synthesis or degradation of particular proteins. The apparent appearance or disappearance of a protein may be due to changes in its solubility. For example, salt extraction of unfertilized as compared to fertilized eggs have shown that more than 10% of the total egg proteins become insoluble at the time of fertilization (*118*). Decreases in the concentration of a number of free amino acids during early cleavage have also been reported (*119*). Recent studies, however, have revealed that extensive and serious autolytic changes occur in aqueous cellular homogenates of marine invertebrate embryos. These *in vitro* changes proceed regardless of low temperatures and nonphysiological pH (*120*). Unless enzymes are first inactivated, autolysis appears to be virtually unavoidable. The artifacts thus produced are of such magnitude as to obliterate the true patterns of chemical changes during development. A technique for the extraction of biological materials which appears to be free of autolytic artifact has been developed for amino acid and protein analyses, but relatively few data obtained by this method are available at present (*120*). An extraction procedure which should inactivate enzymes was used to detect a decrease in RNA content during the early stages of sea urchin egg development (*121*). Similar methodology was used in a study of acid-soluble nucleotides (*122*). Figure 18 shows a decrease in two nucleoside triphosphates during the first 50 hours of development, while the corresponding monophosphates increased. Beginning with the plutei stage, there occurred a successive decrease of all nucleotides and nucleotide bases.

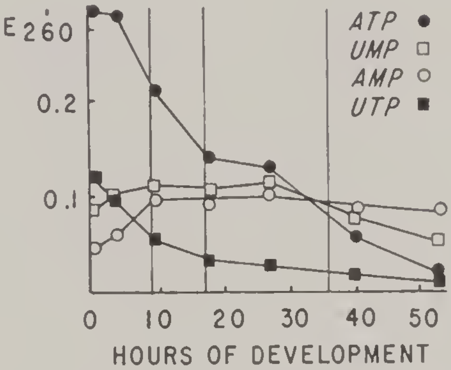

Fig. 18. Acid-soluble nucleotides during sea urchin egg development. From Hultin (*122*).

a. Protein Synthesis. Protein synthesis as a function of developmental stage has been studied in the intact egg by following the incorporation of labeled NH_3, CO_2, and amino acids into protein. From the cleavage stages, it has been found that the nonsoluble protein fraction showed a continuously increasing rate of incorporation. The incorporation of $N^{15}H_4Cl$ into unfertilized and fertilized eggs over a 5-hour period is shown in Fig. 19. It can be seen that a striking difference exists in the two cases with respect to the incorporation of ammonia into the trichloroacetic acid (TCA)-soluble fraction (right ordinate) (*123*). This may be due to changes in permeability which are known to occur on fertilization. By following the rate of osmotic swelling in hypotonic sea water under various conditions, *Arbacia* eggs have been shown to exhibit enhanced permeability on fertilization (*124*). Some very interesting experiments on phosphate accumulation have also shown a marked increase in permeability on fertilization or on artificial parthenogenesis, even in the absence of cleavage (*125*). Because of probable differential labeling during development of the intracellular amino acid precursors of protein synthesis, it is difficult to interpret results such as those shown in Fig. 19. Ideally the intracellular specific radioactivity of an amino acid should be compared to the specific radioactivity of the protein at different stages of development. Apparently it is difficult to free the

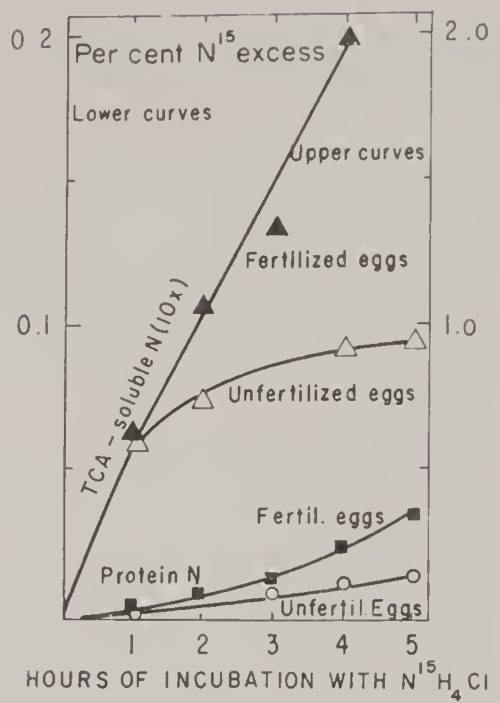

FIG. 19. The incorporation of $N^{15}H_4Cl$ into fertilized and unfertilized sea urchin eggs. From Hultin (*123*).

cells from traces of highly labeled amino acid trapped within the fertilization membrane, blastacoel, etc. (*126*).

Data which are not affected by alterations in permeability during development were obtained by injecting S^{35}-DL-methionine into the body cavity of adult females prior to harvesting the eggs (*127*). After fertilization, the radioactivity, based on nitrogen content, was followed in the TCA-soluble (left figure) and the particulate (right figure) fraction during 18 hours of development. As might be expected, and as Fig. 20

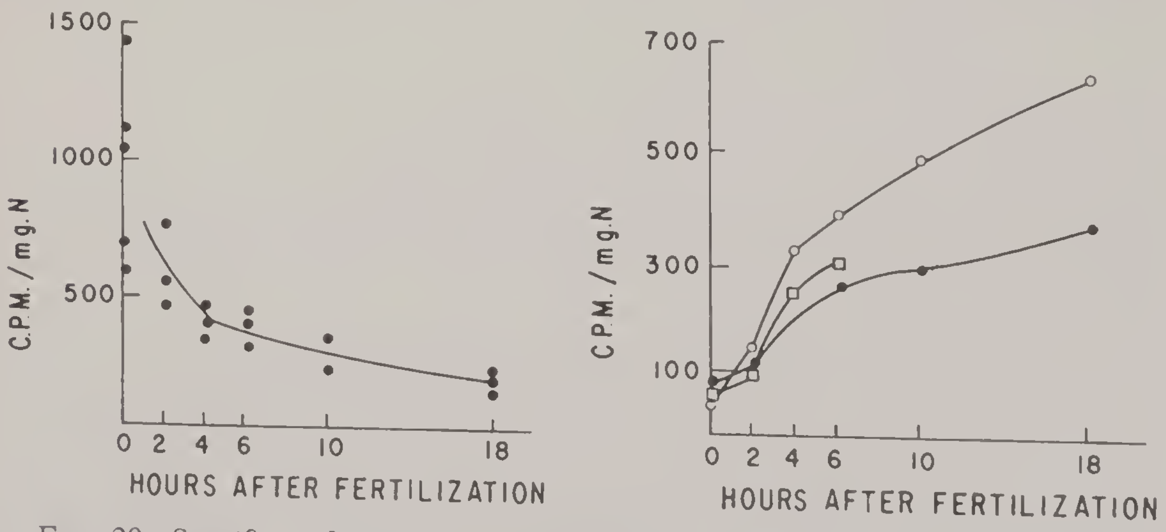

FIG. 20. Specific radioactivity of TCA-soluble (left) and particulate (right) fractions following an injection of S^{35}-DL-methionine. From Nakano and Monroy (*127*).

indicates, the radioactivity of the free methionine pool decreases while the radioactivity of the particulate fraction increases. However, from such data, little can be deduced concerning alterations in the rate of protein synthesis. The total radioactivity of the precursor S^{35}-methionine pool is constantly diminishing, and fluctuations in the concentration of pool methionine (and hence its specific radioactivity) may also be taking place, owing to the liberation of unlabeled methionine from endogenous protein reserves, for example. Again, information is needed on the specific radioactivity of intracellular methionine at the developmental stages examined.

In view of the above reservations concerning data on apparent alterations in the rate of protein synthesis during development, a recent study using mitochondria isolated from sea urchin eggs is of much interest. Mitochondria in good condition, judging from P:O ratios, were prepared before and after fertilization and incubated 2 hours in the presence of a labeled amino acid. The incorporation of label into the mitochondria was shown not to be due to an exchange process, and the major radioactivity present did not depend on terminally bound amino acids (*128*). As Fig. 21 shows, a constant decrease in the ability to incorporate S^{35}-methionine is found, starting from the unfertilized egg through the 40th hour of development of the fertilized egg. These data are at present in apparent conflict with the *in vivo* experiments reported above. In another recent study using various kinds of cell-free preparations, however, greater incorporation of C^{14}-L-leucine into protein was observed in extracts of fertilized as compared to unfertilized eggs (*129*). It was noted that a system containing partially purified particles

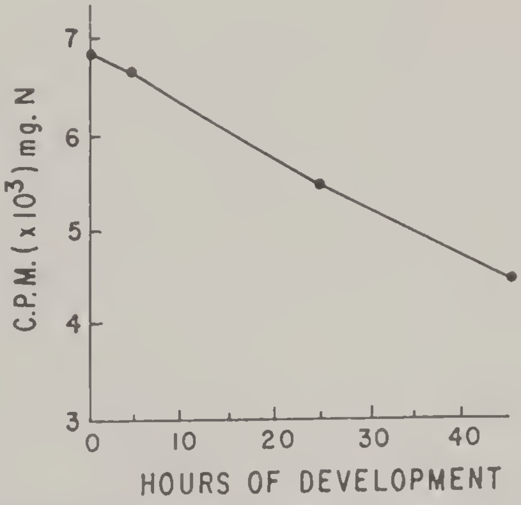

FIG. 21. The incorporation of S^{35}-DL-methionine into mitochondria prepared at different stages of sea urchin egg development. From Giudice (*128*).

gave the smallest differences between fertilized and unfertilized eggs. Perhaps such differences will eventually be ascribed to some extramitochondrial factor.

B. The Double-Gradient Theory

The differentiation of a sea urchin egg must depend upon the evolution of different kinds of biosynthetic processes in different parts of the egg. One end of the egg axis is called the animal pole and is concerned with ectodermal differentiation; the other end is the vegetal pole, and this region controls entomesodermal differentiation. If the vegetal region is removed or damaged (by lack of potassium or sulfate ions), a supernormal development of the animal region occurs. Excessive vegetal differentiation can be induced through damage or inhibition (by lithium ions) of the animal region (*130, 131*). Excessive development of one of these areas can lead to complete extinction of the other. Thus arose the concept of two antagonistic gradients along the egg axis. Removal of the most-animal material, which normally differentiates into the apical tuft, enables the less-animal material to perform this differentiation. Removal of the vegetal skeletal-forming material causes the less-vegetal material to form the skeleton (*132*). Today one might think of mutual feedback inhibition and repression to help explain the basis for such a dual antagonism in the development of biosynthetic abilities. Such phenomena are characteristic of differentiating systems (*133*). A more primitive example can be seen from experiments with the migrating pseudoplasmodium of the slime mold discussed earlier. If the pseudoplasmodium is cut in half, the cells in the foremost third of the presumptive spore segment can now differentiate into presumptive stalk cells. Some of the cells in the former stalk end of the severed pseudoplasmodium now acquire the ability to become spores (*134*).

Various lines of indirect evidence, some of which will be discussed below, have suggested that the ectoderm may develop a stronger mitochondrial activity than the entomesodermal region. Changes in the relative number of Nile blue sulfate-stainable granules (relative mitochondrial density, RMD) during early cleavage and blastula stages are shown in the left-hand curve of Fig. 22. The right-hand curve gives similar data for the blastula and young gastrula stages. In these experiments, sample sectors for counting were chosen by chance with regard to polarity of the egg. Joining these two curves gives an S-shape quite similar to the respiratory curve shown in Fig. 16 (*135*), suggesting a causal relationship. The strong rise in mitochondrial count beginning at the mesenchyme blastula stage is retarded by Li^+ treatment, which inhibits respiration (*136*). An investigation has also been directed to-

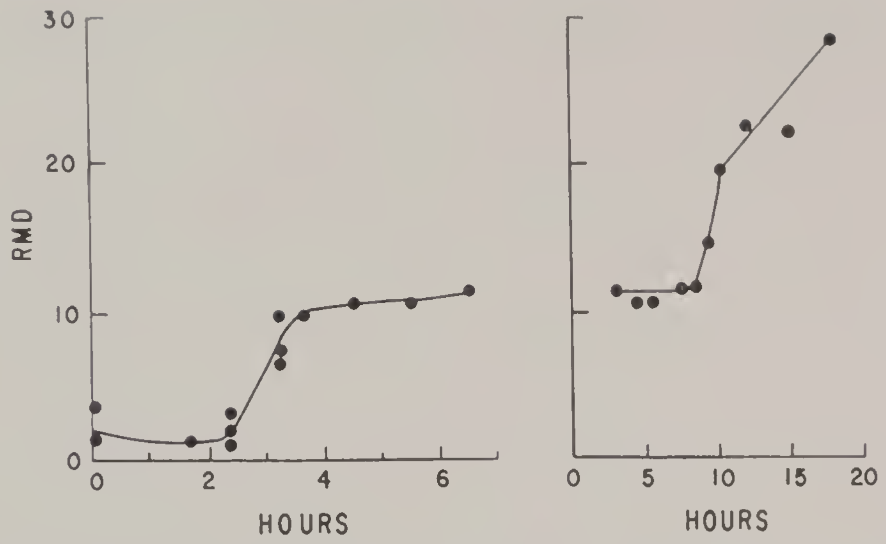

FIG. 22. Changes in the relative mitochondrial density (RMD) during early sea urchin egg development. From Gustafson and Lenicque (*135*).

ward the question of possible differences in mitochondrial activity of the animal pole as compared to the vegetal pole (*137*). Figure 23 shows the mitochondrial density plotted against the distance from the animal pole in normal larvae and in larvae vegetalized by lithium ions. These data, which were obtained at the mesenchyme blastula stage of development, clearly show an animal-vegetal gradient with respect to mitochondrial frequency. This gradient becomes more and more pronounced as differentiation progresses, owing to a sharp rise in mitochondrial number in the animal region as against a quite constant number at the more vegetal pole. The suggestion is made that the vegetal region produces

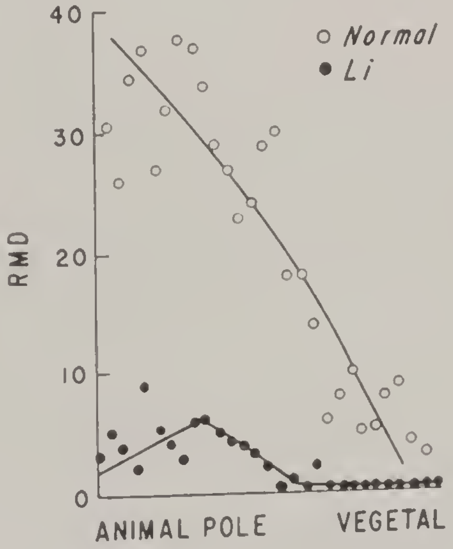

FIG. 23. Mitochondrial density of the animal compared to the vegetal pole. From Gustafson and Lenicque (*137*).

inhibitors of early mitochondrial development. However, another investigation indicates that these Nile blue sulfate-stainable granules may not all represent true mitochondria (see ref. *137a*). Furthermore, this investigation failed to show a gradient distribution of mitochondria in the mesenchyme blastula and gastrula of two species of sea urchin. Also in contrast to the mitochondrial gradient, there is hardly any difference in the oxygen consumption of isolated animal and vegetal halves (*138*).

The relative size of the ectoderm (animal pole) has been correlated with the rise in respiration following fertilization (*136*). Measurements of the respiratory quotient in the period of rising respiration have suggested an increased carbohydrate degradation during this period of differentiation (*139*). The pathway of glucose oxidation will be discussed in the next section.

C. MECHANISMS OF ALTERED ENZYME ACTIVITIES

1. *In Vivo Evidence*

The importance of aerobic metabolism in the early development of the sea urchin egg is evident, since cleavage cannot proceed under anaerobic conditions. Although glycolytic enzymes are present in fertilized *Arbacia* eggs, their low activity is indicated from the fact that almost no lactate is formed either aerobically or anaerobically (*140*). Glucose-6-phosphate is found as a product of glucose metabolism in both egg extracts and intact eggs, a fact indicating the utilization of glucose via a phosphorylating pathway. Experiments on the oxidation of C^{14}-1-glucose and C^{14}-6-glucose by intact *Arbacia* eggs have demonstrated the importance of the TPN shunt pathway in the early stages of development. The ratio of $C^{14}O_2$ from C^{14}-6-glucose:C^{14}-1-glucose was 0.07 in the unfertilized egg, 0.12 25 minutes after fertilization and 0.28 for 24-hour embryos (*141*). These results indicate that glucose is degraded more rapidly via the TPN shunt than by the aldolase step in unfertilized eggs, with the glycolytic pathway becoming quantitatively more important as development proceeds. Similar alterations in the mode of glucose utilization were noted earlier in sporulating slime molds and starving *E. coli* cells.

Consistent with the above role of the hexose monophosphate shunt are some data on the enhanced level of TPNH after fertilization (*142*). Table XIV gives the total pyridine nucleotide content of *Spisula* eggs, expressed in millimicromoles per milliliter of packed cells. In these eggs, as well as in the eggs of *Arbacia*, a three- to sixfold increase in TPNH level was regularly observed subsequent to fertilization. The *absolute*

TABLE XIV
Total Pyridine Nucleotide[a] of *Spisula* Eggs[b]

	Minutes after fertilization	
Nucleotide	Zero	150
DPN	163	140
DPNH	5	11
TPN	11	5
TPNH	9	65

[a] Values in millimicromoles per milliliter of packed cells.
[b] Krane and Crane (*142*).

amount of glucose metabolized via the shunt increases strikingly after fertilization; a high TPNH level is also consistent with the predominant role of this nucleotide in synthetic processes (*143*).

Referring to the earlier discussion of the postulated (albeit tenuous) correlations during early development between the rise in respiration, increased carbohydrate degradation, enhanced mitochondrial density at the animal pole, and differentiation of the ectoderm, it may be mentioned that shunt metabolites have animalizing tendencies and shunt inhibitors have the opposite morphogenetic effect (*144*). Whether these effects could be related to the double-gradient phenomenon remains to be seen.

2. *In Vitro Evidence*

In view of the *in vivo* evidence for increased shunt activity subsequent to fertilization, it is of interest to consider *in vitro* data on the concentration of two shunt enzymes. The activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was examined in homogenates and supernatant fractions under conditions in which the enzyme activity was stable. About 2.4 μmoles of TPN were reduced per minute by the homogenate from 1 gm. of unfertilized or fertilized eggs. TPN reduction by 6-phosphogluconate also gave comparable values for eggs in the two stages of development. Furthermore the relationship of enzyme activity to substrate concentration was the same for both enzymes (*145*). Another indication of comparable enzymatic potential in fertilized and unfertilized eggs comes from data on pentose formation from glucose-6-phosphate during TPN reduction. Table XV demonstrates a similar accumulation of pentose in the presence of the two kinds of extracts.

To estimate the ability of egg extracts to degrade carbohydrate via

TABLE XV
FORMATION OF PENTOSE DURING REDUCTION OF TPN BY SUPERNATANT FRACTION FROM *Arbacia* EGGS[a]

Date	Expt. no.	Stage of development	TPN reduced (μmoles)	Pentose formed (μmoles)
7/23	20W	Unfertilized	0.12	0.07
7/28	24W	Unfertilized	0.16	0.06
7/29	25W	Fertilized	0.14	0.06
8/4	30W	Fertilized	0.15	0.06

[a] From Krahl *et al.* (*145*).

the glycolytic pathway, DPN reduction was measured in the presence of fructose-1,6-diphosphate and ADP. The data of Fig. 24 show that DPN reduction was only a few per cent of the rate of TPN reduction by glucose-6-phosphate, a fact suggesting a relatively low activity for the glycolytic pathway in both fertilized and unfertilized eggs. Curves *A–E* indicate TPN reduction with glucose-6-phosphate, fructose-6-phosphate, 6-phosphogluconate, fructose-1,6-diphosphate, and glucose-1-phosphate, respectively. Curve *F* shows DPN reduction with fructose-1,6-diphosphate as substrate and 0.001 *M* ADP present. The rate of DPN reduction could account for only about one-third of the oxygen consumption of the fertilization eggs. The rate of glucose-6-phosphate oxidation via TPN, however, is sufficient to account for an oxygen consumption of 1700 mm.^{3} per hour per gram of eggs, wet weight. The actual oxygen consumption of fertilized eggs is 310 and of unfertilized

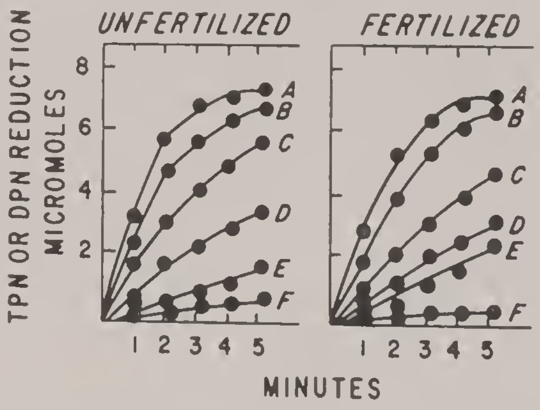

FIG. 24. TPN or DPN reduction with various substrates in extracts prepared before and after fertilization. Curves *A–E:* TPN reduction with, respectively, glucose-6-phosphate, fructose-6-phosphate, 6-phosphogluconate, fructose-1,6-diphosphate, and glucose-1-phosphate. Curve *F:* DPN reduction with fructose-1,6-diphosphate plus 0.001 *M* ADP. From Krahl (*146*).

eggs 70 mm.^{3} per hour per gram eggs (*146*). There is, therefore, in *the presence of excess substrate*, an activity of glucose-6-phosphate dehydrogenase adequate to support a rate of oxygen consumption about six times that observed for the fertilized and twenty-four times that observed for the unfertilized egg. It thus appears that major alterations in respiration and shunt activity are not accompanied by comparable changes in the concentration of two critical enzymes, which are in great excess anyway. As in the case of the slime mold discussed earlier, a simple explanation for the enhanced activity of the hexose monophosphate shunt and, specifically, the two dehydrogenases, is an increased endogenous supply of substrates. The data for pentose formation from glucose-6-phosphate also suggest that substrates, not enzymes, limit the activity of this pathway (Table XV); the amount of enzyme present can form in about 2 hours an amount of ribose equal to that in the total ribonucleic acid of the egg. Another enzyme of carbohydrate metabolism, hexokinase, does not alter significantly in concentration at fertilization (*147*).

Studies on catalase and cytochrome oxidase in sea urchin egg extracts have again emphasized the dangers, *in vitro* and *in vivo*, of equating enzyme activity with enzyme concentration. A number of factors, which may or may not be *in vitro* artifacts, have been shown to influence enzyme activity in homogenates (*148, 149*). Changes in cytochrome oxidase activity during development in two different experiments are shown in Fig. 25. Since this enzyme is largely located in mitochon-

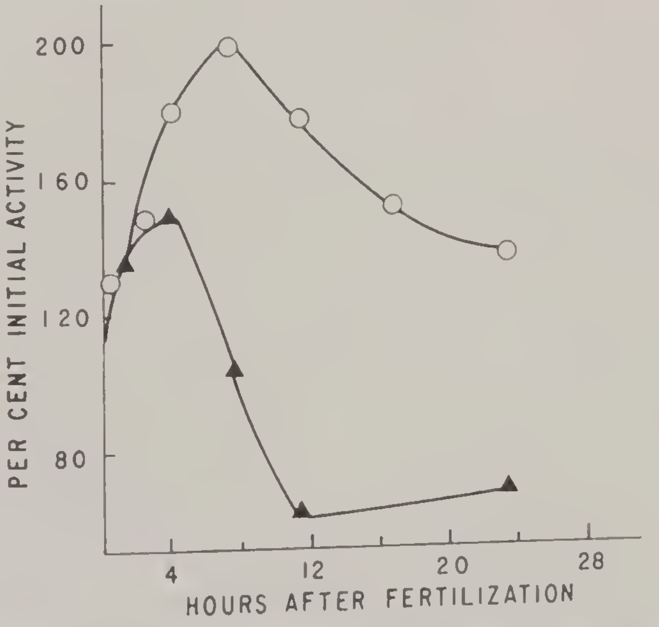

FIG. 25. Alterations in cytochrome oxidase activity during sea urchin development. From Deutch and Gustafson (*149*).

dria, its activity might be expected to rise in parallel to the increases in mitochondrial number (Fig. 22). On the contrary, a sharp drop in the curve is noted after the first few hours of development. The initial rise in activity may be only apparent. The activity of lysates slowly rises on aging in 0.2 *M* phosphate buffer. Dissociation from an inhibitor could be involved, or the phenomenon could reflect enhanced conditions for enzyme-substrate union brought about by a swelling and rupture of the mitochondrial capsule. The drop in cytochrome oxidase activity as development progresses may reflect an increasing lability or inaccessibility of the system, as the enzyme gradually becomes incorporated into the mitochondria (*137*). Similarly, the low activity of the unfertilized egg may reflect a higher degree of manipulative enzymatic destruction during homogenization. (The cytoplasm is less rigid at this stage, and no fertilization membrane is present.) Under certain conditions, aging, freezing, and other treatments result in activity losses. The higher the initial activity of a fresh preparation, the steeper is its decrease on aging. The addition of proteins raises the activity of aged, but not of fresh, preparations.

Catalase activity, in contrast, consistently demonstrates an increased activity after aging for 30 minutes. This was found to be due to the dissociation or destruction of an inhibitor during the assay. The relative activity of catalase as a function of time after fertilization shows a steady drop. These results could be a true reflection of the *in vivo* enzyme activity, or they could reflect the gradual development *in vivo* or *in vitro* of the inhibitor mentioned above. Another interesting example of an inhibitor influencing an enzyme during development has been described in the chick (*150*). A decreased arginase activity in hearts of developing embryos has been correlated with an increased inhibitor activity. The inhibitor appears to be like ribonucleic acid in nature. This system also boasts of a stimulatory agent, protein in nature, which increases the activity, but not the concentration of arginase (*151*). In summary, it is very difficult to interpret data on relative enzyme activities assayed *in vitro* in a system constantly changing physically and chemically as differentiation proceeds.

Other investigations have shown that a number of enzymes associated with mitochondria exhibit a sharp rise in activity at the mesenchyme blastula stage, which also marks the beginning of the second respiratory rise (*152*). This is also the stage at which an increase in mitochondrial number was observed. It is of course tempting to draw conclusions from such a correlation. However, in view of the difficulties inherent in measuring enzyme activities *in vitro*, extensive studies on optimal assay conditions, stability, presence of inhibitors or activators, etc., must first

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be carried out for each enzyme at each developmental stage. Such studies are particularly desirable since some enzymes have been found not to change in concentration, and since the observed *in vivo* alterations in respiration and metabolic pathways could be explained by mechanisms involving changes in enzyme activity not dependent upon changes in enzyme concentration.

VI. Amphibian Differentiation

A. The Energy Requirement of Differentiation

1. *Alterations in Respiration*

One group of workers in this area have found a gradual increase in oxygen consumption during amphibian development (e.g., *153, 154*), and others have reported definite phases of respiratory intensity (*155, 156*). Recent work at low temperatures has given strong support to the conclusion that distinct phases in oxygen consumption do in fact exist. Apparently at high temperatures development proceeds at a rate which obscures small irregularities in the respiratory curve (*156*). Thus in Fig. 26, the curve at 18° shows relatively ill-defined minor peaks. In contrast the upper curve, particularly, derived from data at 10°, exhibits two clear maxima at 44 and 56 days.

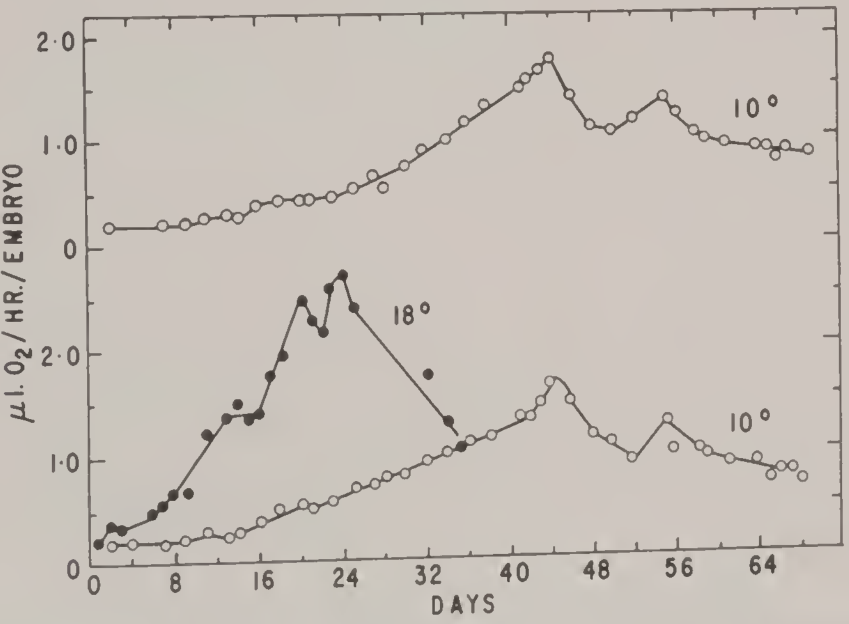

Fig. 26. Respiration during amphibian development at two temperatures. From Løvtrup (*156*).

It has been possible to demonstrate variations in the respiratory rate also at the level of different regions of the gastrula of *Amblystoma punctatum*. This can be seen from 4 experiments in Fig. 27, in which the

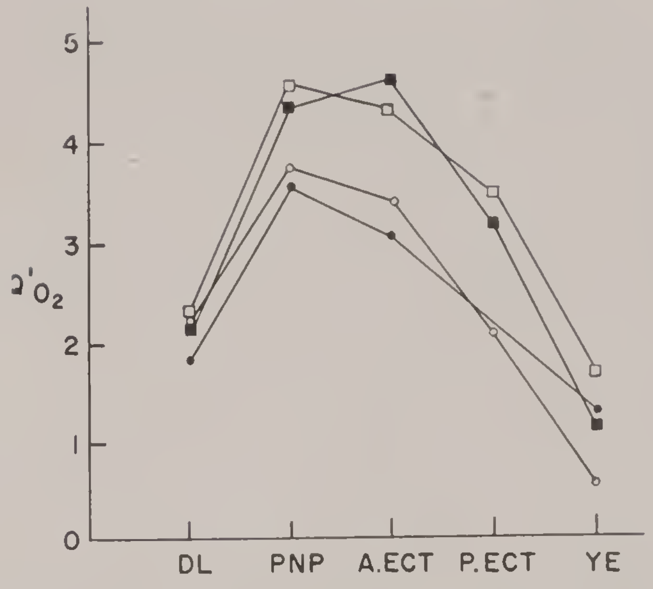

FIG. 27. Respiratory rates in different regions of the gastrula of *Amblystoma punctatum.* DL, dorsal lip; *PNP*, presumptive neural plate; *A.ECT*, ectoderm from animal pole; *P.ECT*, ectoderm from near vegetal pole on ventral side; *YE*, yolk ectoderm. From Boell (*157*).

region of the gastrula is indicated along the abscissa (*157*). The gastrula stage occurs in the first few days after fertilization, during the gradually increasing phase of respiration.

2. *Utilization of Endogenous Reserves*

Alterations during development in the respiration of the whole organism, as well as variations within the organism at the gastrula stage of development, have been correlated with the utilization of endogenous reserve materials. Figure 28 indicates the sequence of utilization of endogenous carbohydrate, fat, and protein in the axolotl during development (*158*). It has been shown that the R.Q. of intaet amphibian eggs averages about 0.6 during cleavage and rises abruptly to 1.0 during gastrulation (*159*). Carbohydrate utilization during this period is located in the dorsal area around the blastopore. Table XVI gives R.Q. data

TABLE XVI
RESPIRATORY QUOTIENTS OF DORSAL LIP (DL) AND VENTRAL ECTODERM (VE)[a]

Investigator	Material	R.Q. DL	R.Q. VE
Brachet	*Discoglossus*	1.02	0.73
Brachet	*Rana fusca*	0.97	0.80
Boell, Koch, and Needham	*Amblystoma mexicanum*	0.98	0.87
Needham, Rogers, and Shen	*Rana temporaria*	0.92	0.81

[a] From Boell (*157*).

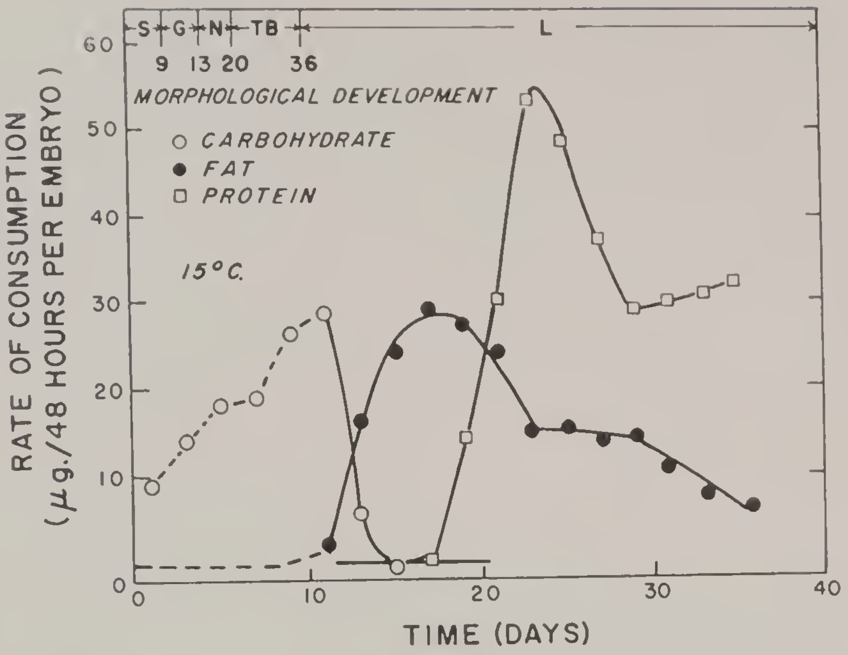

FIG. 28. Utilization of endogenous reserves during amphibian development. From Løvtrup (*158*).

from four different investigations comparing the dorsal lip and ventral ectoderm of the amphibian gastrula. Values for the glycogen content of different regions in the embryo at the gastrula compared to the blastula stage are given in Table XVII. All these data, taken together, indicate

TABLE XVII
TOTAL GLYCOGEN IN THE AXOLOTL EMBRYO[a,b]

Region	Blastula	Gastrula	% Decrease
D1	17.8	16.5	7
D2	12.0	8.3	31
D3 + V3	4.3	3.9	9
V1	16.7	16.5	1
V2	10.0	9.3	7

[a] Values in milligrams per cent dry weight.
[b] From Heatley and Lindahl (*160*).

an intense carbohydrate utilization in the dorsal area at the time of gastrulation. The second phase of carbohydrate utilization presumably reflects metabolic activity in the remaining parts of the animal region. This phase corresponds in time with the differentiation of this part of the embryo.

The first horizontal phase of the respiratory curve at 18° (Fig. 26) begins at about 12 hours, when carbohydrate utilization diminishes and fat consumption begins (see Fig. 28). The first maximum in respiration (18 hours at 18°) always coincides with the maximum in the rate of fat consumption. The second maximum usually corresponds fairly closely to the maximum in the rate of protein utilization. During the phase of decreasing respiration, energy is supplied almost exclusively by protein. Two facts clearly contribute to the complexity of the respiratory curves: (i) the energy-requiring differentiation processes in the embryo begin at different times in different regions of the embryo; and (ii) within each region there is a succession of energy sources utilized.

In frog eggs, yolk platelets occupy almost half the volume of the cell. These platelets are composed of phosphoprotein bound to lipid and are a major energy source during development. In the intact egg this reserve material remains fairly stable throughout the entire winter. Rupture of the egg, however, results in an immediate and powerful autolytic reaction (*161*). Figure 29 shows the release of orthophosphate (curve 2) from protein phosphate (curve 3) in an ovarian homogenate at pH 5.0. Curve *1* represents total acid-soluble phosphate and curve *4* lipoid phosphate. The pH optimum of this reaction is quite sharp; conceivably the process of fertilization promotes an acidity sufficient to activate these autolytic enzymes.

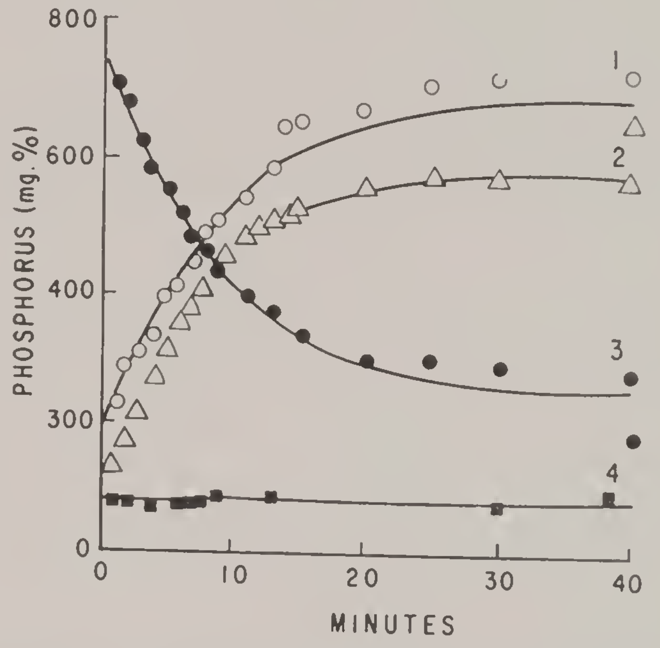

FIG. 29. The release of orthophosphate (curve 2) from protein phosphate (curve 3) in ovarian homogenates of the amphibian. Curve *1*, total acid-soluble phosphate; curve *4*, lipoid phosphate. From Harris (*161*).

Phosphoprotein phosphatase activity has also been studied in protein fractions isolated from frog embryos at different stages of development and from animal as compared to vegetal regions of the gastrula. Whole extracts containing both enzymes and phosphoproteins show a steady rise in phosphoprotein phosphatase activity from cleavage through hatching, after which a decline in activity coinciding with disappearance of visible yolk is observed (*162*). Whole extracts from vegetal regions split phosphoprotein at a rate 400% greater than that measured in animal regions. However, when enzyme fractions are removed from these regions and given equal amounts of substrate, animal regions exceed vegetal regions in enzyme activity. Thus in all probability differential enzyme activity *in vivo* has been achieved by differential substrate localization.

a. Alterations in Protein Patterns. A good deal of evidence supports the viewpoint that new antigens appear during development [e.g., see (*163, 164*)]. An electrophoretic analysis has been made of the serum proteins of *Rana catesbeiana* at various stages of development (*165*). The results indicate that metamorphosis results in a redistribution of the globins in favor of the slower- and faster-moving components at the expense of globins of intermediate mobility.

In another recent study, a number of electrophoretically distinguished protein fractions were followed in developing embryos and compared to adult organ proteins. The results are summarized in Table XVIII, which shows various proteins in the adult organ extracts which were present in the unfertilized egg extract. The first band, Fl, fluoresced, and the others stained with bromophenol blue. These data suggest that most if not all the proteins extracted from various adult organs are present in the unfertilized egg (*166*). In considering these data, however, it should be mentioned that the methodology used could have resulted in autolytic artifacts.

TABLE XVIII

Bromophenol Blue-Stained Proteins of Adult Organ Extracts Present in Unfertilized Egg Extracts[a]

	Band				
Adult organ extract	Fl	B	A	H	J
Intestine	–	–	–	–	±
Brain	–	–	–	+	+
Skeletal muscle	+	–	–	+	+
Blood	–	+	+	–	±

[a] From Spiegel (*166*).

Results such as those recorded in Table XVIII raise the interesting possibility that, during differentiation, structural changes occur in a protein which result in active enzyme as well as different solubility characteristics. Another possibility is that differential losses occur in various cell types with respect to their ability to synthesize a protein. Support for this hypothesis can also be found in the synthesis of cardiac myosin in the chick embryo (*167*). Early in development there is a uniform distribution of cardiac myosin, whereas later this synthetic ability is restricted to the presumptive heart cells. Another very interesting example in the chick is the behavior of alkaline phosphatase (*168*). In the first week of incubation, this enzyme is moderately active throughout the various tissues, but it increases or disappears as functional differentiation of particular organs occurs.

B. Mechanisms of Altered Enzyme Activities

If a brei of fertilized frog eggs is prepared, respiration increases compared to that of the intact eggs. The phenomenon is illustrated in Fig. 30 (*159*). This so-called "injury metabolism" can be observed in sea urchins (*169*), insects (*170*), and plants (*171*).

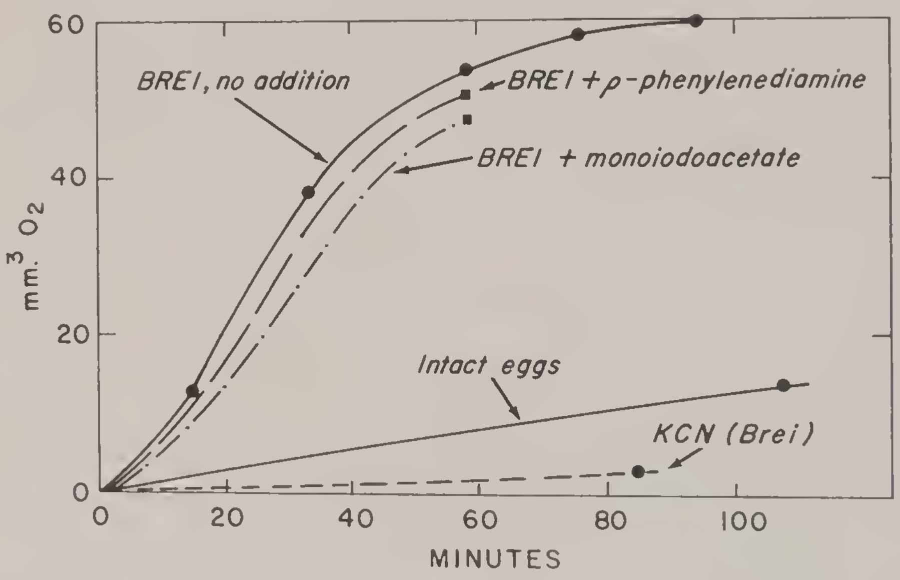

Fig. 30. Respiration of a brei of frogs' eggs compared to that in intact eggs. From Brachet (*159*).

One must conclude from data such as those presented in Fig. 30 that the egg normally contains an excess of respiratory enzymes. It is of interest that a brei of tadpoles has a lower rate of oxygen consumption, whereas the intact tadpoles have a more intense respiration than the intact eggs. These observations would be compatible with an increasing availability of oxidizable materials *in vivo* as development proceeds.

Many investigations have dealt with *in vitro* studies on alterations in enzymatic activities during amphibian development. Examples of these and other biochemical changes that occur are given in Fig. 31 and 32

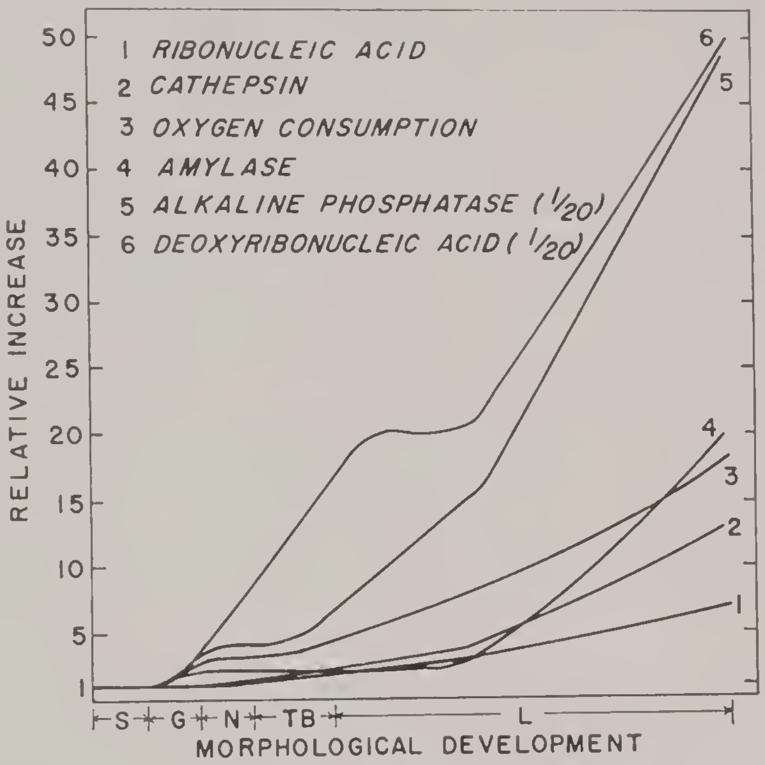

FIG. 31. Biochemical changes that occur before the initiation of larval development of the amphibian. From Løvtrup (*172*).

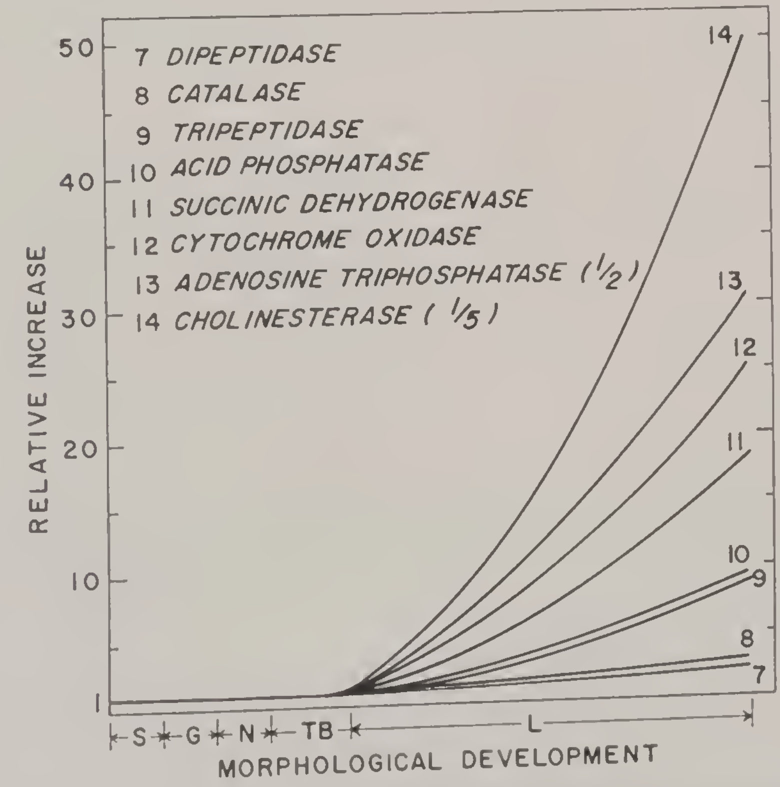

FIG. 32. Alterations in enzymatic activities that occur after the initiation of larval development. From Løvtrup (*172*).

(*172*). The first group includes respiration, as well as substances that increase in activity and/or amount before larval development. Figure 32 depicts enzymatic alterations detected only after the initiation of larval development. Note the lack of correlation in time between the initial enhanced respiration and the increase in succinic dehydrogenase and cytochrome oxidase activities (*173*). Other studies, in contrast, have described significant increases in these enzyme activities during early development (*174*). However, even in these cases the cytochrome oxidase is at all times greatly in excess of that needed to sustain the respiratory needs of the embryos. The concentrations of these enzymes therefore do not limit respiration during early amphibian differentiation. In general, quite different enzyme patterns during development have been obtained by various workers (*175, 176*); a consistent picture is not yet obvious.

1. *Cholinesterase*

The relatively late appearance of cholinesterase activity is to be expected, since this "specialized" enzyme is concerned with neural tissue, which does not become differentiated until early in larval development. The activity of this enzyme, which is thought to play an essential role in synaptic transmission, bears a close relationship to neural differentiation. In the amphibian, functional differentiation of various parts of the central nervous system occurs at different stages of embryonic development. From the motor behavior of the developing animal, it might be predicted that neural differentiation begins at the spinal cord and proceeds sequentially cephalad to the hindbrain (*HB*), midbrain (*MB*), and forebrain (*FB*). Not only the order of appearance of cholinesterase activity, but also the final level of activity at the end of the prefeeding period of development varies progressively from posterior to anterior. Another indication of the specificity and significance of this enzyme is its dissociation from general metabolic activities. Figure 33 compares cholinesterase and respiratory activities in various regions of the brain. The patterns of these two activities are clearly dissimilar. In Fig. 34, the activity of cholinesterase and succioxidase in relation to total protein content is shown during the course of normal development (*177*). Whereas the respiratory enzyme closely parallels the growth curve, cholinesterase exhibits a striking increase during the same period of development. These data give strong support to the conclusion that there is a dependent relationship between cholinesterase activity and the process of neural differentiation. Although this enzyme is an unusually good example of one specifically associated with a particular differentiation process, the mechanisms underlying its enhanced activity are as yet unknown.

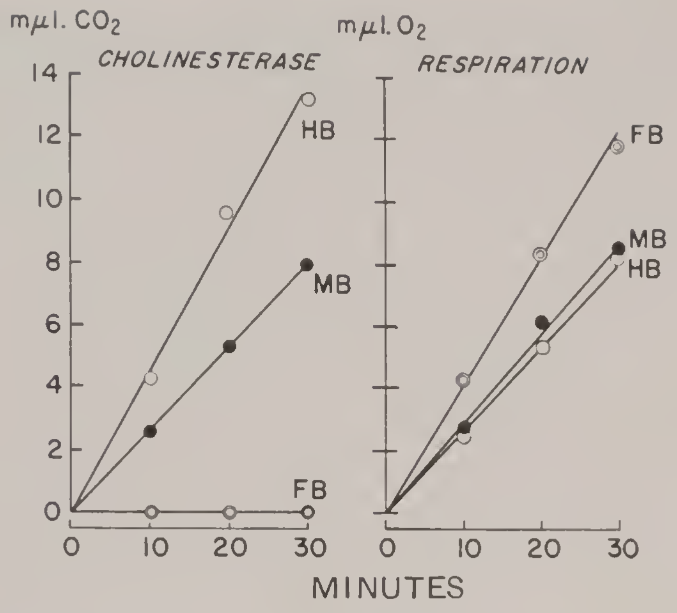

FIG. 33. Cholinesterase and respiratory activities in various regions of the amphibian brain: *HB*, hindbrain; *MB*, midbrain; *FB*, forebrain. From Shen (*177*).

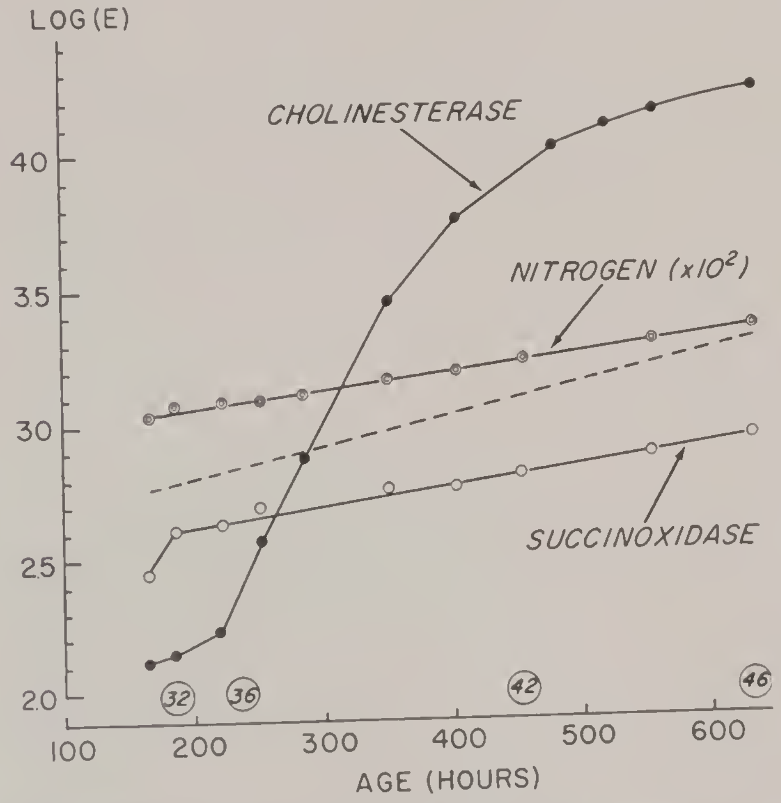

FIG. 34. Cholinesterase and succinoxidase activity in relation to the total protein content during amphibian development. Shen (*177*).

2. *Carbamyl Phosphate Synthetase*

Amphibian metamorphosis represents in part a preparation for the transition from an aquatic to a terrestrial habitat. This conversion necessitates numerous anatomical and biochemical changes, including a change-over from ammonotelism to ureotelism. The essential enzymatic alterations involved here are the enhanced activities of the Krebs-Henseleit urea cycle enzymes. Urea is synthesized in the liver according to the sequence of reactions shown in Eqs. 1–5.

$$\text{Ammonia} + \text{Bicarbonate} + 2\text{ATP} \xrightarrow[\substack{\text{acetyl glutamate} \\ \text{Mg}^{2+}}]{\substack{\text{carbamyl phosphate} \\ \text{synthetase}}} \text{Carbamyl phosphate} + 2\text{ADP} + \text{P}_i \qquad (1)$$

$$\text{Carbamyl phosphate} + \text{Ornithine} \xrightarrow[\text{transcarbamylase}]{\text{ornithine}} \text{Citrulline} + \text{P}_i \qquad (2)$$

$$\text{Citrulline} + \text{Aspartate} + \text{ATP} \xrightleftharpoons[\text{enzyme (Mg}^{2+})]{\text{condensing}} \text{Argininosuccinate} + \text{AMP} + \text{PP}_i \qquad (3)$$

$$\text{Argininosuccinate} \xrightleftharpoons[\text{enzyme}]{\text{cleavage}} \text{Arginine} + \text{Fumarate} \qquad (4)$$

$$\text{Arginine} + \text{H}_2\text{O} \xrightarrow[\text{Mn}^{2+}]{\text{arginase}} \text{Urea} + \text{Ornithine} \qquad (5)$$

Significant increases occur during metamorphosis in the activity of these enzymes (*178, 179*) as well as liver ATPase and glucose-6-phosphatase (*180*), and both acid and alkaline phosphatases (*181*). On the other hand, liver succinoxidase activity as well as endogenous respiration decreases, indicating a degree of selectivity with respect to enhanced enzyme activities during this period of development.

Particular emphasis has been placed on a study of carbamyl phosphate synthetase. Its activity as a function of developmental stage number is shown in Fig. 35. The specific activity (enzyme units per

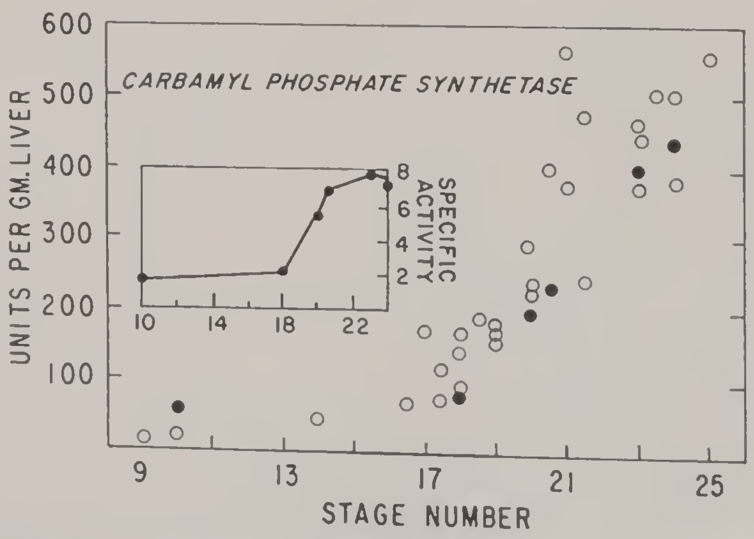

FIG. 35. Carbamyl phosphate synthetase activity as a function of amphibian development. From Brown *et al.* (*178*).

milligram protein) has increased as much as tenfold over a corresponding stage number range in other experiments. No indication of the existence of inhibitors or activators during the assay was found (*178*). Although unlikely, it is conceivable that the observed differences in activity of this enzyme *in vitro* could have occurred through differential destruction during its preparation at the earlier as compared to the later stages. That the enhanced enzyme activity in extracts prepared at different developmental stages actually reflects differences in enzyme concentration has been carefully demonstrated by means of immunochemical and isotope techniques. Antibody which was 100% precipitable by frog carbamyl phosphate synthetase was isolated from rabbit and goat antisera to this enzyme (*182*). It was shown that the ratio of enzyme nitrogen determined immunologically to the enzymatic activity is the same for the extract from the livers of tadpoles before, as compared to after, metamorphosis. In other words, no preformed, immunologically active, enzymatically inactive precursor was present.

The purified antibody was then used to isolate carbamyl phosphate synthetase from tadpole livers after the following experiment was performed (*183*): *Rana clamitans* tadpoles were preincubated for 1, 2, 3, 4, 6, 9, and 14 days at 22–23° in water containing L-thyroxine (0.02 μg. per milliliter). They were then treated by intraperitoneal injection with 0.025 ml. of leucine-C^{14}, and incubated further in thyroxine for 22 hours prior to isolation of the enzyme. In Fig. 36, the filled circles represent

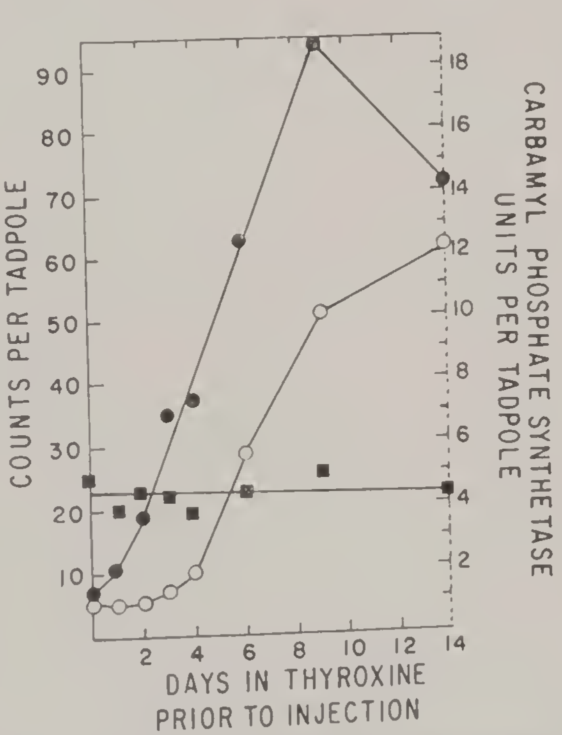

FIG. 36. The incorporation of C^{14}-leucine into enzyme and nonenzyme protein during thyroxine treatment. From Metzenberg *et al.* (*183*).

counts incorporated into carbamyl phosphate synthetase per tadpole; the filled squares represent counts incorporated per tadpole into combined "noncarbamyl phosphate synthetase" protein fractions (collected during the purification procedure). The open circles indicate enzyme units per tadpole. The constancy of incorporation into the "noncarbamyl phosphate synthetase" fractions demonstrates the existence of a large class of proteins not affected by thyroxine. Because of its different behavior with respect to leucine-C^{14} incorporation, this class of proteins could not contribute a preformed, immunologically different, enzymatically inactive precursor of carbamyl phosphate synthetase. The authors conclude that thyroxine induces the synthesis *de novo* of the enzyme. However, another possible interpretation of these data is that no change in the rate of synthesis of this enzyme has in fact been induced by thyroxine. The incorporation of leucine-C^{14} proceeds linearly *beginning with* the control animals not treated with thyroxine. The increase in counts per tadpole is comparable to the increase in enzyme concentration. These results would be compatible with an unaltered rate of synthesis accompanied by a decreased rate of breakdown of the enzyme (either *in vivo* or *in vitro*). In fact, the initial lag in enzyme accumulation compared to the linear incorporation of counts into the enzyme suggests a high rate of enzyme turnover in the absence of its accumulation, as the authors point out. It is quite possible that carbamyl phosphate synthetase has an intrinsically high rate of synthesis and breakdown and that thyroxine in some way increases the steady state level of the enzyme without affecting its rate of synthesis.

VII. Summary and Speculation

A. THE INITIATION AND CONTROL OF DIFFERENTIATION

The initiation of development can be brought about by a great variety of "trigger mechanisms," including, for example, starvation in the cellular slime molds, hydration in seeds, and fertilization in the sea urchin. However, regardless of the stimulus, a common result is the degradation of endogenous reserves. This pattern of metabolism is a necessity as an energy source in the case of the slime molds, which can differentiate in the complete absence of exogenous nutrition. Thus this organism offers a striking example of the conflict between the multiplication of a cell at optimal rates and its morphogenesis.

Since the transformation of one cell type into another frequently occurs under nutritionally poor conditions, the major energy source available necessarily is of endogenous origin. We have seen that under starvation conditions even cells which do not differentiate (e.g., *Escherichia coli,* and morphological mutants of *Dictyostelium discoideum*) break down their macromolecules. These observations suggest that this degradative activity, while probably necessary to the process of differentiation, is not uniquely correlated with or caused by differentiation. An example of a possible stimulant for the breakdown of endogenous macromolecules would be the initial utilization of "feedback inhibitors" of proteolytic action. Some support for the existence of this mechanism can be found in the reports that a source of soluble nitrogen inhibits proteolysis in *E. coli* and in plant seedlings.

The stimulation of spore germination by alanine offers an unusual example of the utilization of endogenous material during development. In this system, it was found that most of the alanine degraded was actually of endogenous origin. It was further demonstrated that the oxidation of this compound is necessary in order for germination to proceed. Mn^{2+} stimulates germination more indirectly by activating proteolytic enzymes, which in turn release oxidizable intermediates. At a more complex level of development, sperm extracts of the sea urchin contain proteolytic enzymes, and proteases of the egg become activated at the time of fertilization.

Perhaps the clearest examples of the sequential utilization of endogenous materials can be seen in seed and amphibian development. In these cases, alterations in respiratory intensity and respiratory quotient values have been carefully correlated with chemical analyses demonstrating the sequential disappearance of macromolecules and the appearance of oxidizable substrates as development progresses.

All data considered, it appears that one important factor limiting morphogenesis is the availability of sufficient endogenous substrates for the energy and building-block requirements of differentiation.

B. Mechanisms of Altered Enzyme Activities

Major alterations in the activity of specific enzymes must necessarily be involved in the metabolic changes associated with differentiation. In many instances these alterations are of a quantitative nature and are difficult to assess accurately *in vitro.* A basic problem lies in the fact that the physical-chemical environment of an enzyme *in vivo* and *in vitro* changes rapidly in a differentiating organism. Evidence for the absence of an enzyme or for alterations in the concentration of an enzyme during development must be accepted with caution. In extracts of the slime mold and castor bean for example, certain enzymes are rapidly inactivated by heat-labile factor(s) at one stage of development and not at another. Hexokinase was initially thought to be present in vegetative cells but not in spores of aerobic bacilli. It then became apparent that this enzyme is very labile in spore extracts and must be protected by glucose in order to detect its activity. Enzyme lability may be due to proteases, which appear to be very active in the early stages of differentiating systems. Proteolytic activity has been shown to give rise to serious autolytic artifacts in the protein patterns seen in extracts prepared at different developmental stages of the sea urchin egg. Evidence from studies with the amphibian and sea urchin has indicated alterations in the solubility of pre-existing proteins during development. Another type of difficulty can and has confused the interpretation of *in vivo* isotope incorporation experiments. Striking alterations in the permeability of inorganic ions and amino acids have been observed during development in the sea urchin and slime mold. A valid comparison of the specific radioactivity of a protein fraction or enzyme labeled early and late in development depends on a knowledge of the specific radioactivity of the immediate precursor being incorporated. This information is often very difficult to obtain, particularly in more complex systems. Many of the available data on alterations in the rate of protein synthesis during development are difficult to interpret for such reasons.

It is generally true that metabolites and not enzymes limit biochemical reactions *in vivo*. The accumulated evidence indicates that in some cases alterations in the availability and the utilization of endogenous metabolites are responsible for alterations in the activity of particular enzymes during development. *In vivo* evidence demonstrated a striking increase in the metabolism of glucose via the hexose monophosphate shunt after fertilization in the sea urchin. An examination *in vitro* of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity showed them to be the same before as after fertilization. Furthermore, their activity was adequate to support a rate of oxygen consumption manyfold higher than that observed for the unfertilized egg. Thus major alterations in respiration and shunt activity were not accompanied by comparable changes in the concentration of two critical enzymes, which are in great excess anyway. Cytochrome oxidase behavior represents another example of this type in both sea urchin and amphibian development. The simplest explanation for these observations is an enhanced availability of endogenous substrates. More direct evidence for the role of substrates during development in determining enzyme activity is found for phosphoprotein utilization in the vegetal and animal regions of the amphibian egg. In this case, differential enzyme activity is due to differential substrate localization, not to a gradient in enzyme concentration.

Activation of a preformed enzyme may occur in the glucose-oxidizing system of bacterial spores. There is also evidence for enzyme activation of catalase in the sea urchin and arginase in the chick. Specific inhibitors appear to affect the activity of cytochrome oxidase in the sea urchin and arginase in the chick. Since these inhibitors are macromolecular in nature, their effects are not examples of feedback control of an enzyme by a product of its activity. End product control of the activity or synthesis of an enzyme is considered to be a major mechanism by which a cell achieves its remarkable balance of cellular components. In many instances at least, biochemical differentiation has been shown to involve quantitative alterations in particular enzymatic activities. It is quite probable that many such biochemical changes will eventually be recognized to be under end product regulation. At present the cases of "feedback" in differentiating systems are on a rather descriptive level. One example is the inhibition of the development of spores by stalk cells (and vice versa) in the slime mold. Another example is the "double gradient" of the sea urchin, in which the animal and vegetal pole are antagonistic to each other's development. Although effects such as these are not yet understandable in biochemical terms, they may well represent very complex cases of end product regulation of metabolism.

Alterations in the activity of an enzyme do not necessarily imply alterations in the concentration of this enzyme. Even more important to emphasize is the fact that alterations in enzyme concentration do not necessarily imply alterations in the rate of synthesis of this enzyme. It would be of fundamental interest to know whether the rate of synthesizing a specific protein changes during the process of differentiation.

In order to answer this important question, it is first necessary to find a system in which the increase in activity of an enzyme is due to an increase in its concentration *in vivo*, but not due, for example, to the removal of an inhibitor, or to *in vitro* differential enzyme stability, extractability, etc., in extracts prepared at various developmental stages. An increase in enzyme concentration (of *in vivo* origin) could occur through: (*a*) the release or activation of a preformed precursor; (*b*) an increased stability and hence accumulation of the enzyme; (*c*) a relatively greater utilization and decrease of nonenzyme proteins suggesting an apparent increase in concentration of the enzyme; (*d*) an increase in the rate of synthesis of the enzyme. (Of course, more than one of these mechanisms could be operating at once.)

Results such as those for carbamyl phosphate synthetase in the amphibian demonstrate that the enhanced enzyme activity in extracts prepared at successive developmental stages is in fact due to a difference in enzyme concentration. The labeling data indicate that the enzyme did not arise from a preformed precursor. However, such data do not allow a choice between mechanisms (*b*) and (*d*). In order to distinguish between these two possibilities, the pulse of labeled amino acid must be so short as to label only a small fraction of the existing enzyme pool, and the specific radioactivity of the purified enzyme should be determined early and late in development (*184*). If under these circumstances the specific radioactivity of the enzyme is the same early and late in development, then an increase in the rate of synthesis is responsible for the higher enzyme level. If enhanced breakdown *in vivo* is responsible for the low enzyme level early in development, the enzyme will have a higher specific radioactivity at this stage than it will have late in development. The data suggest that the latter could be the case for carbamyl phosphate synthetase (*183*).

In conclusion, the process of differentiation may be initiated in a number of ways. Once initiated, however, certain biochemical features seem to be common to all the systems discussed. These include alterations in respiration correlated with the utilization of endogenous macromolecules, and changes in the activity of certain enzymes. Operational artifacts such as the differential *in vitro* stability of an enzyme at various stages of development make difficult interpretations of data indicating increases in the concentration of an enzyme during morphogenesis. In some cases, observed changes in enzyme activities are not accompanied by changes in enzyme concentration, and are apparently due to the increased availability of endogenous substrate to the enzyme.

Acknowledgment

For helpful criticisms of relevant sections of the manuscript I wish to thank Dr. E. R. Stadtman, Dr. M. Krichevsky, Dr. M. Krahl, and Dr. H. Halvorson.

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CHAPTER 2

Comparative Aspects of Metabolic Control*

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I. Introduction 73
II. Control of Enzyme Activity 74
  A. Feedback Inhibition in Bacteria 74
  B. Feedback Inhibition in Other Organisms 77
  C. Product Inhibition 78
  D. Control of the Activity of Energy-Supplying Pathways . . . 79
III. Control of Enzyme Formation 81
  A. Variations in Enzyme Amount 81
  B. Enzyme Induction 82
  C. Enzyme Repression 95
IV. Activation of Enzymes 103
V. Discussion 105
  References 107
  Note Added in Page Proof 118

I. Introduction

The myriad of chemical reactions that go on in a living cell do not proceed at random rates but, rather, are adjusted to meet the biological needs of the organism. This article will consider some of the mechanisms by which these adjustments are effected and will examine them from the viewpoint of comparative biochemistry. The nature of such metabolic control mechanisms has already been reviewed (*1*).

The first step in a comparative study is to assemble data on control mechanisms from as many taxonomic groups and cell types as possible. Such a survey has not been made before. Then, upon examining the data, contributions of several kinds might emerge. As discussed by the

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* Aided by a grant, E3277, from the United States Public Health Service.

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Editors of this treatise in Volume I, Chapter 1, these could include contributions to our knowledge about (*a*) the mechanisms by which control processes operate, (*b*) the evolutionary relationships of certain taxonomic groups, (*c*) the evolution of control mechanisms, and (*d*) the correlation of types of control mechanisms with the environment of different cells or organisms.

We restrict our discussion of control mechanisms in the following ways. First, we deal only with control mechanisms that operate within a single cell; intercellular control mechanisms, such as hormonal controls, are excluded. The second area that will not be discussed here has to do with mutation and selection. This mechanism is certainly one of the ways in which the metabolism of a species is adjusted to its environment. For example, if a reaction is too rapid, mutants could be selected in which the structure of an enzyme is altered in such a way that its catalytic activity is reduced. Or, mutants could be selected in which the amount of the enzyme is lower than in the original type. These genetic mechanisms of metabolic control are to be distinguished from the phenotypic mechanisms of metabolic control which we are to discuss.

As implied in the previous paragraph, phenotypic control mechanisms are of two sorts: those affecting the catalytic activity of specific enzymes and those affecting their amount. These mechanisms will now be discussed in turn.

II. Control of Enzyme Activity

A. Feedback Inhibition in Bacteria

It became recognized in the early 1950's that the activity of enzymes in biosynthetic pathways of the intestinal bacterium *Escherichia coli* might be controlled by feedback mechanisms. At this time knowledge of the intermediates in many of these pathways was advancing rapidly. In order to understand how these feedback inhibitions operate, let us consider a model biosynthetic pathway (2)

$$A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow F \rightarrow G \rightarrow H$$

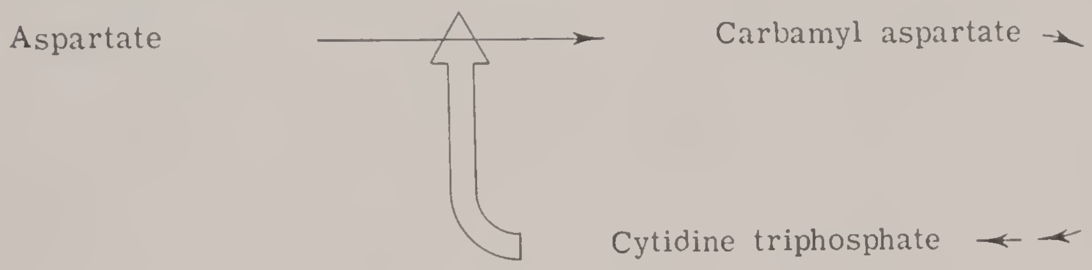
by which the nutrient A is converted through the intermediates B, C, D, E, F, and G to the amino acid (or nucleic acid base) H, each step in the pathway being catalyzed by a separate specific enzyme. Suppose now that there is a mutant which lacks one enzyme of the pathway, say the enzyme for converting D to E. Because of this enzymatic deficiency the mutant cannot synthesize the amino acid H and, therefore, needs a dietary source of this amino acid for growth. It is not surprising that D can be synthesized when this mutant is put into a medium lacking H.

What is surprising is that when H is added to the medium not only is the ability to grow restored, but the synthesis of D is immediately severely inhibited. The end product H is a feedback inhibitor of the synthesis of D and therefore of H itself. This curious ability of the end product to prevent intermediates before the genetic block from being synthesized has been observed in the biosynthetic pathways of several amino acids and nucleic acid bases [for a review, see (*3*)]. Furthermore, it could be shown in some cases that the effect of the end product could not be attributed to the fact that it enabled the mutant to resume growing. This made it likely that the end product inhibited in a fairly direct manner the activity of an early enzyme in its own biosynthetic pathway.

The second important line of evidence involved in the discovery of feedback control of enzyme activity came from isotopic competition experiments with wild-type (nonmutant) bacteria (*4*). Let us take as an example an experiment with *E. coli* growing exponentially in minimal medium containing C^{14}-glucose as the only carbon source. If, for example, C^{12}-threonine is added to the medium and the radioactivity of the amino acids appearing within a generation time in the bacterial proteins is measured, threonine is found to have a specific activity much lower than that of other amino acids (except isoleucine which is synthesized in part from threonine). Thus, much of the threonine incorporated into protein comes from the medium in such an experiment. The prevailing interpretation of such findings (until 1955) was that the C^{12}-threonine (in this example) entered the intracellular pool of amino acids and thereby diluted the internally synthesized C^{14}-threonine (*4*).

Now according to this interpretation, the internally synthesized C^{14}-threonine that is prevented from appearing in the cell proteins should appear somewhere else. However, this threonine could not be found either in the cells or in the medium, nor did detectable amounts of C^{14}-intermediates in threonine's biosynthetic pathway appear anywhere. Hence the prevailing interpretation could not be correct; threonine does not merely dilute the internal threonine pool, it must also inhibit the internal synthesis of threonine at some early step. Similar conclusions were generally reached when the synthesis of other amino acids from C^{14}-glucose was studied in the presence of the corresponding C^{12}-amino acids (*4*), although it must be noted that there were some exceptions, principally glutamate and aspartate, where the results could be explained largely by simple isotopic dilution.

One explanation of the mutant and isotopic experiments with intact bacteria would be that an amino acid or nucleic acid base, despite its apparent lack of chemical resemblance to the substrates or products of early enzymes in its biosynthetic pathway, directly inhibits the activity of one of these enzymes. This hypothesis was tested by Yates and Pardee (*5*) and Umbarger (*6*) working with cell-free extracts. In confirmation of the hypothesis, Yates and Pardee showed that in cell-free extracts of *E. coli* cytidylic acid inhibits the activity of aspartate transcarbamylase, the first enzyme in the pyrimidine pathway. Later work showed that cytidine triphosphate is an even better inhibitor (*7*); see Scheme I.



(I)

Similarly, Umbarger showed that when isoleucine is added to *E. coli* extracts it inhibits the activity of threonine deaminase, the first enzyme unique to isoleucine's biosynthetic pathway (*6, 8*).

The results could not be due in either case to a mass action effect of the end product transmitted back through the biosynthetic chain of reactions to the first enzyme because there is an irreversible step in each pathway. Moreover, in the pyrimidine case, the inhibitory effect of the end product is observable also with mutants (in whole cells or extracts) in which one of the enzymes in the pathway is missing.

Comparable findings have since been made in the biosynthetic pathways of several other amino acids, for example, valine (*9*), threonine (*10*), tryptophan (*11, 12*), and histidine (*11, 13, 14*) [for a review, see (*15*)]. Considered together, then, the results of these cell-free experiments complement the mutant and isotopic experiments done on intact cells and show that the end product of a biosynthetic pathway can generally inhibit the activity of a very early enzyme in the pathway.

Nevertheless, it will be noted that even the demonstration of these feedbacks in crude extracts does not prove beyond doubt that the inhibition is direct, especially in view of the fact that the end product usually has little chemical resemblance to the substrates or products of the early enzyme. With the advent of a highly purified preparation of crystalline aspartate transcarbamylase (*16*), it has recently become possible to test whether the inhibition is really direct. The test showed without doubt that cytidine triphosphate can inhibit the enzyme as well in the purified state as in crude extracts (*7*). This encourages us to assume that similarly direct inhibitions underlie the feedbacks affecting the activity of other biosynthetic enzymes in extracts and intact cells.

Up to this point, all the work on biosynthetic feedbacks that we have discussed concerns pathways that synthesize major end products. One wonders whether there are similar feedbacks in the biosynthetic pathways of trace cellular constituents, such as vitamins or coenzymes. Recent experiments suggest that if there are any feedbacks affecting the activity of flavin-synthesizing enzymes then they must be much weaker than the feedbacks in large-scale pathways (*17*).

B. FEEDBACK INHIBITION IN OTHER ORGANISMS

The information about feedbacks presented so far has come from work on *E. coli, Aerobacter aerogenes,* and *Salmonella typhimurium,* species of three related genera in the family Enterobacteriaceae (coliform bacteria). However, all the techniques used to demonstrate feedbacks in these organisms would seem readily applicable to other bacteria and to many fungi, algae, and protozoa. Experiments with extracts could readily be applied also to multicellular organisms, but it may be harder in these cases to devise *in vivo* tests. It is reasonable then to inquire whether such feedbacks occur in organisms outside the family Enterobacteriaceae.

Only one example has been reported in noncoliform bacteria; in this case, arginine inhibits both the synthesis of the biosynthetic intermediate ornithine by nongrowing suspensions of *Micrococcus glutamicus* and the phosphorylation of *N*-acetyl glutamate, an early enzymatic step in arginine biosynthesis, by cell-free extracts of this organism (*17a*).

No cases of feedback inhibition are known from protozoa, algae, or multicellular land plants (metaphytes). However, a few brief studies have been made with two ascomycete fungi, *Neurospora crassa* and *Saccharomyces cerevisiae* (bakers' yeast). Threonine was found to inhibit the activity of homoserine kinase, the first distinctive enzyme of threonine biosynthesis, not only in *E. coli* extracts but also in extracts of bakers' yeast. Threonine is a slightly better inhibitor of the *E. coli* enzyme than of the yeast enzyme (*10*). There is preliminary evidence that in *Neurospora* tryptophan can inhibit the activity of an early enzyme in its biosynthetic pathway (*18*). Some effects that isoleucine and valine have on the excretion of biosynthetic intermediates by *Neurospora* probably can be accounted for by feedback inhibition (*19*) of enzyme activity or of enzyme formation (see Section III, C). Histidine inhibits the activity of the first enzyme of the histidine pathway in *Saccharomyces* extracts (*19a*), and histidine's effects in *Neurospora* might also be explained in the same way (*20*).

Several examples of feedback have been found in warm-blooded vertebrates. Purine nucleotides are strong inhibitors of the activity of the first enzyme of purine synthesis in extracts of pigeon liver, and also of the partially purified enzyme (*21*). Experiments with intact ascites tumor cells in mice also suggest that adenine (and certain purine thiols) can inhibit the activity of enzymes that catalyze early steps in the purine biosynthetic pathway (*22, 23*). Other experiments with crude extracts of mouse ascites tumor cells show that end products of the pyrimidine pathway inhibit the activity of both the first enzyme, aspartate transcarbamylase, and the second enzyme, dihydro-orotase (*24*). The inhibitory effect of uracil on orotic acid excretion by a child afflicted with a rare disease (*25*) could be due to feedback inhibition of the activity of early enzymes in the pyrimidine pathway or of the formation of these enzymes (see Section III, C). Preliminary results from work with chick embryo extracts (*26*) and intact mammalian cells (*27*) suggest that the formation of pyrimidine deoxyribonucleotides from pyrimidine ribonucleotides is controlled by some sort of feedback inhibition. Finally, there is evidence that bile acids prevent their own formation from cholesterol both in living rats (*28*) and when added to a cell-free preparation from rat liver (*29*).

In conclusion, the examples cited, although scanty, are diverse enough to suggest that feedbacks affecting enzyme activity are widespread among different phyla.

C. Product Inhibition

Some enzyme-catalyzed reactions are inhibited by their products to a greater degree than would be expected according to the law of mass action. For example, the phosphorylation of glucose catalyzed by rat hexokinase is inhibited by the reaction product, glucose-6-phosphate, at concentrations that are far too low to reverse the reaction appreciably (*30–32*). A list of such product inhibitions has been made recently (*33*).

Product inhibition can occur in biosynthetic pathways, for example the pyrimidine pathway. Thus, uridylate inhibits the conversion of orotidylate to uridylate in extracts of rat liver (*34*), although this reaction is essentially irreversible, at least in *Saccharomyces* (*35*). Orotidylate strongly inhibits the conversion of orotate to orotidylate in cell-free preparations of *Saccharomyces* (*35*) and probably also in *E. coli* (*36*). This inhibition can occur in intact bacteria as well, since an *E. coli* mutant that lacks orotidylate decarboxylase excretes primarily orotate rather than orotidylate when starved of pyrimidines (*36*). There are indications that product inhibition occurs in a variety of other biosynthetic pathways as well, since adenosine diphosphate inhibits glutamine synthetase in extracts of sheep brain (*37*) and glutathione synthetase in extracts of pigeon liver (*38*) and serine inhibits phosphoserine phosphatase, the last enzyme of serine biosynthesis, in extracts of chicken (*38a*) and rat liver (*38b*). In addition, nicotinic acid mononucleotide inhibits nicotinic acid mononucleotide pyrophosphorylase, an enzyme of DPN synthesis in beef liver (*38c*).

Some enzymes that are not known to be part of a biosynthetic pathway are also inhibited by their products. Inorganic phosphate inhibits phosphatases from a variety of organisms including bacteria of several families (*39–42*), fungi (*43*), and placental mammals (*44–46*), although in a few cases this may be owing to reversal of the reaction by mass action. Indole inhibits tryptophanase in *E. coli* (*47*). Adenosine diphosphate inhibits the fructokinase of rat and cattle liver (*48*) and the hexokinase of rat tissues and *Aspergillus oryzae* (an ascomycete), but not yeast hexokinase (*31*, *49*). The hexokinase of rat tissues and calf brain is also inhibited by glucose-6-phosphate, but the yeast (*30–32*) and *Aspergillus* (*49*) enzymes are not inhibited.

It is possible that product inhibition acts as a control mechanism, like feedback inhibition, but in most cases this remains to be ascertained by suitable experiments with intact organisms. The product inhibition of mammalian hexokinase is an exceptional case because there is some evidence that it does function in life as a control mechanism (*50*).

D. Control of the Activity of Energy-Supplying Pathways

Special control mechanisms of the types discussed so far have not been shown to play an important part in adjusting the rates of energy-supplying pathways to changing physiological needs. The latter may be controlled rather simply by mass-action effects of substrates such as adenosine diphosphate (ADP) and inorganic phosphate. For example, if adenosine triphosphate (ATP) production begins to exceed physiological needs, the ATP concentration would necessarily build up and the ADP and inorganic phosphate concentrations would fall. As ADP and inorganic phosphate are needed for ATP production, the rate at which ATP is produced will automatically decline under such circumstances (*51*).

Special control mechanisms may, nevertheless, have some role in controlling the activity of enzymes in these pathways. Tracer experiments on intact ascites cells from mice suggest that hexokinase activity, and perhaps, therefore, the rate of glycolysis, may be governed by product inhibition (*50*). This may not, however, be a general mechanism of regulating this reaction or glycolysis because, as mentioned above, hexokinase is not subject to product inhibition in nonmammalian organisms such as bakers' yeast. A possibly analogous control in the

Krebs cycle may arise from the fact that oxalacetate can inhibit succinic dehydrogenase in rat tissues (*52–54*). This inhibition seems to be widespread as it occurs also in the avocado (*55*), a seed plant. Carbon dioxide also inhibits succinic dehydrogenase of castor beans (*56*). Whether these inhibitions function as control mechanisms *in vivo* remains to be ascertained.

As well as control mechanisms within an energy-supplying pathway, there may be mechanisms that regulate the relative rates of alternative pathways of energy supply. The best known of these is the Pasteur effect, the inhibition of glycolysis by oxygen, which regulates the relative rates of respiration and glycolysis. The most recent of many reviews on this subject is by Aisenberg (*57*).

The mechanism by which oxygen inhibits glycolysis has received a great deal of attention. For a time it was widely thought to be a simple competition for ADP or inorganic phosphate between glycolysis and the oxidative phosphorylation system of respiration. A Pasteur effect would arise if oxidative phosphorylation could go on at lower concentrations of ADP or inorganic phosphate than glycolysis could. But this model has proved too simple to explain all the data now available on the Pasteur effect in yeast and vertebrate tissues, and so more complex versions have been proposed [see (*57*), for a discussion of these]. Oxygen may act differently in lactic acid bacteria because oxygen uptake may not be accompanied by oxidative phosphorylation in these organisms (*58*), yet some of them exhibit a Pasteur effect (*59*). Furthermore, oxygen may not act via oxidative phosphorylation in pea-seed extracts; in this case oxygen inactivates triosephosphate dehydrogenase (*60*), one of the glycolytic enzymes, but it is not known whether oxygen would act in this way in intact plants. In conclusion, the mechanism of the Pasteur effect has not yet been established in any organism and, as pointed out by Aisenberg (*57*) and Turner (*61*) its mechanisms may not be the same in different organisms.

The adaptive value of the Pasteur effect is to conserve energy-supplying carbohydrates. This is evident from the fact that, although glycolysis can occur both aerobically and anaerobically and respiration can occur only aerobically, glycolysis supplies energy at least ten times less efficiently (per glucose utilized) than respiration. In the presence of oxygen it is, therefore, more economical to get energy from carbohydrate by respiration than by glycolysis.

As implied in several reviews, the Pasteur effect is taxonomically widespread. It occurs in the tissues of several vertebrates (*59, 62*) and in a variety of mollusks (*63*), annelids (*64*), and some parasitic nematode worms (*65*). However, there is no Pasteur effect in many other parasitic nematodes or in parasitic flatworms (trematodes) although they can use oxygen (*65*). The Pasteur effect occurs in some seed plants (*61*), in bakers' yeast, and in some bacteria, e.g., lactic and propionic acid bacteria (*59*) and *E. coli* (*65a*). There is no doubt, then, that the Pasteur effect is taxonomically widespread; but in view of the enormous amount of work done on carbohydrate breakdown in microorganisms it is somewhat surprising that reports of the Pasteur effect seem rather uncommon.

Regulations analogous to the Pasteur effect occur in some other pairs of pathways. Thus succinate, an intermediate in the Krebs cycle, inhibits the activity of isocitratase, which is a key enzyme of the glyoxylate cycle (*66*). A further mechanism by which the Krebs cycle can inhibit the glyoxylate cycle will be discussed below (Section III, C). Another possible instance of this type of phenomenon may arise from the fact that phosphoglucoisomerase activity is inhibited by 6-phosphogluconate or erythrose-4-phosphate, which are intermediates in the hexose monophosphate shunt pathway (*67*, *68*), but the regulatory importance of this remains to be ascertained.

III. Control of Enzyme Formation

A. VARIATIONS IN ENZYME AMOUNT

The number of functional molecules of an enzyme per cell can vary according to the nutrient conditions. Such changes in enzyme amount provide a means of controlling metabolism that is distinct from the regulation of the rate at which enzyme molecules function. An optimal amount of an enzyme should permit products to be made at a rate that matches the requirements of the cell, whereas insufficient or excessive amounts of enzyme would give rise to problems of supplementation or waste.

Several mechanisms are known that cause variations in the amounts of enzymes. Two of these affect the rates at which specific enzymes are synthesized. The first and best known is enzyme induction, defined as the specific stimulation of enzyme synthesis by a nutrient, usually the substrate. The second is enzyme repression, in which a nutrient or metabolite specifically inhibits the formation of an enzyme. Further, if an enzyme is unstable, its rate of breakdown (or inactivation) could be modified by nutrient conditions; as a result its amount could vary. Finally, the amount of functional enzyme can change because an inactive form of the enzyme (zymogen) is activated or a tightly bound intracellular inhibitor is removed.

There are, in addition, apparent changes in enzyme amounts that can be attributed to faulty assay conditions. Among these are assays of whole cells, in which a change in apparent amount of enzyme may be observed because of a change in the cell's permeability or changes in the amounts of cofactors of the enzyme.

These factors which cause real or apparent changes in enzyme amount will be discussed in this and the following section. As will be seen, considerable uncertainty often exists as to the mechanism of many of the observed changes.

B. Enzyme Induction

1. *General Concepts of Induction*

Many enzymes are known whose appearance is elicited only when certain specific compounds, usually substrates, are present in the environment. For example, wild-type *E. coli* will grow on a great variety of media without exhibiting more than a trace of β-galactosidase, but when a β-galactoside has been present in the growth medium for a few hours the galactosidase activity of the bacteria is as much as a thousand times higher.

The appearance of enzyme is not due to a selection of mutants which make the enzyme constitutively, i.e., under all conditions. In the first place the appearance of enzyme activity and the achievement of maximum specific activity take place too fast to be accounted for by selection. Conversely, on subculture in the absence of the galactoside, the activity is diluted out too fast to be accounted for by selection. Third, the phenomenon can be demonstrated in nongrowing cultures where there could be no selection (*69*). Fourth, by means of more elaborate techniques, the enzyme can be shown to appear simultaneously in nearly all cells of the culture (*70*).

If the appearance of a specific enzyme in response to the presence of its substrate or a related compound, and in the absence of genetic change, is due to synthesis of the enzyme, the phenomenon is called enzyme induction. Enzymes appearing in this way are known as inducible enzymes. The names formerly used were enzyme adaptation and adaptive enzymes. The concepts are dealt with in numerous reviews [for example, see (*71*)].

The criteria used to establish whether the appearance of an enzyme is due to its synthesis are illustrated by the induction of β-galactosidase in *E. coli*. Indirect evidence is provided by the use of specific inhibitors of the synthesis of macromolecules, for example, chloramphenicol (Chloromycetin) (*72–74*), 5-methyltryptophan (*75*), or ultraviolet light (*76, 77*). When *E. coli* is treated with such agents, β-galactosidase production is inhibited in a few minutes at most (*78, 79*). The same effect is produced by taking out of the medium an amino acid that is needed for growth of an amino acid-requiring mutant (*80*). One can also test the effects of amino acid analogs that are incorporated into proteins during protein synthesis, e.g., *p*-fluorophenylalanine, β-2-thienylalanine, ethionine, 7-azatryptophan, and tryptazan, because enzymes that contain such analogs are sometimes inactive (*81*). These indirect methods all agree in showing that protein synthesis is required for induction.

However, it does not necessarily follow from such evidence that the protein the synthesis of which is required for the appearance of an enzyme is the enzyme itself. This is strikingly illustrated by the fact that the rise in deoxyribonuclease (DNase) activity which takes place when phage DNA enters *E. coli* is prevented by β-thienylalanine and chloramphenicol (*82*), even though the rise does not represent synthesis of the enzyme. The only reason DNase activity rises when phage DNA enters is that a specific intracellular inhibitor of pre-existing enzyme becomes destroyed (*83*). Apparently it is the destruction of inhibitor that requires protein synthesis.

Direct methods are needed in order to show conclusively that the substrate-induced appearance of an enzyme is due to synthesis of the enzyme, i.e., methods that measure enzyme amount rather than enzyme activity. The appearance of β-galactosidase activity is paralleled quantitatively by the appearance of a protein that is immunochemically indistinguishable from highly purified galactosidase. Hence the enzyme could not arise from a protein precursor (zymogen) that has an immunochemical resemblance to the enzyme (*69*). Tracer methods have confirmed this and have shown, furthermore, that the enzyme is formed directly from free amino acids, not from pre-existing proteins of any kind (*84, 85*). Thus, the effect of the galactoside is to induce the formation of the enzyme protein β-galactosidase. The effect could not result from inhibition of breakdown of the enzyme because the enzyme is completely stable and tracer studies also show that there is virtually no protein turnover in growing *E. coli* whether a galactoside is present or not.

2. *Enzyme Induction in Bacteria*

Besides *E. coli* β-galactosidase, many other bacterial enzymes appear in response to substrates or related compounds. With respect to substrate specificity or type of reaction catalyzed, their variety is great, including hydrolases, transglycosidases, kinases, isomerases, decarboxylases, oxidases, and dehydrogenases (*86*). Most of them are catabolic enzymes, but as will be seen later at least one biosynthetic enzyme is inducible by its substrate.

From the taxonomic or ecological standpoint, the variety of species in which substrate-induced enzyme appearance has been reported is also great. To date the eubacteria have provided all the known instances, possibly because little biochemical work of any kind has yet been done on other taxonomic groups of bacteria (e.g., myxobacteria, spirochetes). Many of these instances are from coliform bacteria, but the phenomenon has been reported also in most of the other eubacterial families, for example, Pseudomonadaceae (*87–90*), Nitrobacteriaceae (*91*), Athiorhodaceae (*92, 93*), Thiorhodaceae (*94*), Bacillaceae (*71, 95*), Azotobacteriaceae (*96*), Achromobacteriaceae (*97, 98*), Parvobacteriaceae (*99–101*), Micrococcaceae (*66, 102, 103*), Lactobacteriaceae (*95, 104–107*), Corynebacteriaceae (*108, 109*), Mycobacteriaceae (*110*), Actinomycetaceae (*111, 112*), and Streptomycetaceae (*113–115*). Not all these reports rule out the possibility that the enzyme studied appeared in the culture because of selection of mutants. But even if we disregard such reports (for example, those concerning the corynebacteria and streptomycetes) there is still good reason to think that all eubacteria have control mechanisms which enable certain enzymes to appear when their substrates are present in the medium.

So far the impression may have been gained that a substrate elicits the appearance of only one enzyme; but studies on *Pseudomonas* have led to the realization that this may not generally be true. Like the other eubacteria, *Pseudomonas* can adapt to compounds for which only one enzymatic step is needed to convert them to metabolic intermediates in the main stream of metabolism. Certain carbohydrates and amino acids are examples of such compounds. But *Pseudomonas* can also adapt to aromatic and heterocyclic compounds whose metabolism requires a long sequence of extra enzymes. In such cases the added compound causes all the enzymes in this special pathway to appear (*87, 88*). Similar examples, not involving such long pathways, have since been found in other bacteria, such as *E. coli*, in which galactose brings about the appearance of a sequence of three enzymes needed for the conversion of galactose to glycolytic intermediates (*116*). Another example occurs in *Micrococcus denitrificans*, where carbon dioxide can make several, perhaps many, enzymes of the Calvin cycle appear (*66*).

There is evidence that substrate-induced enzyme appearances in bacteria are often due to enzyme synthesis, i.e., enzyme inductions. Table I illustrates the variety of bacterial families and genera for which such evidence has been obtained. As the table shows, the dependence of enzyme appearance on protein synthesis (indirect test) is widely spread among the eubacterial families. Conclusive direct evidence, however, has been obtained in only three cases: β-galactosidase of *E. coli,* penicillinase of *Bacillus cereus,* and catalase of *Rhodopseudomonas spheroides;* nevertheless, they represent three different families.

3. *Permease Induction in Bacteria*

Systems that actively transport substances into bacterial cells are sometimes inducible. For example, when *E. coli* is first exposed to a β-galactoside, the galactoside can enter the cells only by diffusion, but after a short lag period the cells acquire an active transport system specific for the uptake and accumulation of the galactoside and certain other sterically related galactosides. The appearance of the transport system is not due to selection of mutants. It is inhibited by agents that specifically prevent protein synthesis—indirect evidence that the transport system contains an inducible protein. The name permease is applied to the system, the suffix *-ase* being intended to imply that it is an enzyme because of its marked stereospecificity (*122, 123*). The galactoside permease is distinct from β-galactosidase itself, although both are induced by β-galactosides.

Inducible permeases in *E. coli* are now known for other carbohydrates, e.g., β-glucuronides (*124*) and some amino acids, e.g., tryptophan (*125*). In *Pseudomonas* species there are distinct permeases for each of several Krebs cycle acids (*126–128*), and even for each of the tartaric acid isomers (*129*). *Aerobacter aerogenes* has an inducible permease for citrate (*130*). Inducible permeases for Krebs cycle acids have also been found in *Corynebacterium* (*131*) and *Azotobacter* (*132–134*). Like inducible enzymes, then, inducible permeases are widespread among eubacterial famlies.

Permease induction can lead to mistaken claims of the existence of enzyme adaptation if assays are not conducted on cell-free preparations. One needs only to recall the well-known example of the Krebs cycle enzymes. These enzymes were at first thought to be adaptive in *Pseudomonas* and *Azotobacter* because manometric assays, performed on intact cells, revealed no enzyme activity unless the cells had been grown previously in the presence of added Krebs cycle acids. However, when cell-free preparations were used for the enzyme assays, these enzymes were found to exhibit the same activity per cell whether or not the cells had been grown with the acids (*126, 127, 132*). The adaptation was due not to enzyme formation but to permease formation (*126, 127, 134*).

In view of these results, it would be advisable to remain cautious about the ten examples labeled with the superscript *d* in Table I,

TABLE I

TAXONOMIC DISTRIBUTION OF ENZYME INDUCTION AMONG BACTERIAL GENERA AND FAMILIES[a]

Taxonomic group	Enzyme	Indirect evidence[b]	Direct evidence[c]	Key reference
Enterobacteriaceae				
Escherichia coli	β-Galactosidase	+	+	See text
Aerobacter aerogenes	Glycerol dehydrogenase	+	–	*(117)*
Bacterium cadaveris	Lysine decarboxylase	+[d]	–	*(118, 119)*
Paracolobactrum aerogenoides	Histidase	+[d]	–	*(120)*
Pseudomonadaceae				
Pseudomonas ovalis	Carboxydismutase	+	–	*(90)*
Nitrobacteriaceae				
Hydrogenomonas facilis	Hydrogenase	+[d]	–	*(91)*
Athiorhodaceae				
Rhodopseudomonas spheroides	Catalase	+	+	*(93)*
Bacillaceae				
Bacillus cereus	Penicillinase	+	+	*(121)*
Achromobacteriaceae				
Achromobacter sp.	Cytochrome a_2	+	–	*(97)*
Flavobacterium sp.	Systems oxidizing aromatic acids	+[d]	–	*(98)*
Parvobacteriaceae				
Pasteurella pestis	Krebs cycle enzymes	+[d]	–	*(101)*
Micrococcaceae				
Micrococcus pyogenes	Penicillinase	+	–	*(103)*
Staphylococcus aureus	β-Galactosidase	+[d]	–	*(102)*

Lactobacteriaceae				
Lactobacillus arabinosus	Malic enzyme	+^{d}	—	*(107)*
Leuconostoc mesenteroides	Malic enzyme	+^{d}	—	*(107)*
Streptococcus faecalis	Malic enzyme	+^{d}	—	*(107)*
Actinomycetaceae				
Nocardia corallina	Thymine-uracil oxidase	+^{d}	—	*(112)*

[a] Only the most thoroughly established example of enzyme induction is given for each genus.

[b] Enzyme appearance is prevented by specific inhibitors of protein synthesis.

[c] Enzyme appearance is due to *de novo* synthesis of the enzyme detected by immunochemical or tracer-incorporation methods.

[d] Enzyme assay was conducted with whole cells.

because the demonstration that these adaptations require protein synthesis was based on enzyme assays with whole cells. In these examples, part or all of the requirement for protein synthesis could be for permease induction.

4. *Induction in Other Organisms*

Having discussed induction in bacteria, we can now examine the evidence concerning its existence in other organisms.

a. Algae, Protozoa, and Fungi. Of the seven algal phyla (*135*), five have yielded instances of induction-like phenomena. It was shown many years ago that hydrogenase appears in a variety of algal phyla, including Cyanophyta (blue-green algae), Rhodophyta (red algae), Phaeophyta (brown algae), and Chlorophyta (green algae), after exposure to an atmosphere of hydrogen (*136–138*). This was termed an adaptation when discovered and has since been referred to as an induction (*138a*). However, recent evidence suggests that the enzyme is present in an inactive form before the cells are exposed to hydrogen. Thus, chloramphenicol does not inhibit the appearance of hydrogenase in *Scenedesmus* although it very probably inhibits protein synthesis in this system because it strongly inhibits the incorporation of radioactive carbon into the hot-methanol-insoluble fraction of the cells (*139*). Moreover, the appearance of hydrogenase in *Scenedesmus* is greatly accelerated in the presence of hydrosulfite (*139*), and in *Chlamydomonas* its appearance is apparently instantaneous (*139a*). It is therefore likely that the appearance of hydrogenase is the result of some sort of activation process.

Carboxydismutase is found in extracts of *Chlorella variegata* (Chlorophyta) when grown photosynthetically but not when grown in the dark (*140*). Likewise the green flagellate *Euglena* (Euglenophyta) exhibits carboxydismutase and TPN-linked triosephosphate-dehydrogenase activities only when grown photosynthetically (*140*). The appearance of the dehydrogenase upon exposure of *Euglena* to light is too fast to be accounted for by selection of mutants (*141*). As some photosynthetic species among Cyanophyta, Chrysophyta (e.g., chrysomonads and diatoms) and Pyrrophyta (e.g., dinoflagellates) as well as among Chlorophyta and Euglenophyta can grow heterotrophically in the dark (*142–148*), one guesses that the inducibility of these two photosynthetic enzymes may be widespread. The fact that carboxydismutase is induced by light in a facultatively photosynthetic bacterium (*149*) gives support to this guess.

Some of the enzymes which algae use for heterotrophic growth may also be inducible. When *Euglena* has grown on ethanol as the sole carbon source, the ability of whole cells to oxidize acetate improves by a factor of two over the course of a few hours in buffer plus acetate; this might be attributed to permease or enzyme induction (*149a*). Another example is provided by the enzyme system involved in the oxidation of short-chain fatty acids by *Polytoma uvella*, an obligately heterotrophic relative of the photosynthetic genus *Chlamydomonas* (Chlorophyta). This enzyme system appears when acetate-grown cells are exposed to butyrate. The process is too fast to be accounted for by selection and is inhibited by ultraviolet light in doses that do not affect enzyme activity, a result which suggests that butyrate induces the actual synthesis of the enzyme (*150*); however, induction of a permease could be responsible, as the enzyme was assayed with whole cells.

There is little evidence yet concerning whether induction-like phenomena are common in protozoa, although many species of protozoa are now in pure culture and can use a variety of carbohydrates for growth (*151, 152*), sometimes diauxically (*153*). Besides the examples referred to in *Euglena* and *Polytoma*, which are considered as algae by botanists and as protozoa by zoologists, there is only one preliminary report of an induction-like phenomenon in an undisputed protozoan. In this case, acetate raises the level of isocitratase and malate synthetase, enzymes needed for the net conversion of acetate to carbohydrate, in the ciliate *Tetrahymena* (*153a*). The nutritional adaptations reported earlier in *Tetrahymena* (*154–156*) are obscure in nature and might be due to selection.

Among fungi, which lead a saprophytic life like bacteria, substrates commonly elicit the appearance of enzymes. Examples of such enzymes include cellulase (*115, 157, 158*), pectinesterase and polygalacturonase (*157, 159*), saccharase (*157, 160*), respiratory enzymes (*161*), α-glucosidase (*162, 163*), β-glucosidase (*164–166*), β-galactosidase (*157, 167–169*), adenase (*170*), uricase (*171*), nitrate reductase (*172*), protocatechuic oxidase (*173*), γ-aminobutyrate transaminase (*174*), kynureninase (*175*), tyrosinase (*176*), and enzymes that attack xylose, lignin, and dextran (*157*). Although this list is not complete it includes examples from many species representing more than a score of genera scattered among the three main classes of fungi, namely, phycomycetes, ascomycetes, and basidiomycetes.

It has been shown in several cases that the appearance of fungal enzymes does not result from selection of mutants. Such examples include the appearance of respiratory enzymes (*161*), cytochrome peroxidase (*177*), catalase (*178*), α-glucosidase (*162, 163, 179*), β-glucosidase (*164, 165*), and β-galactosidase (*169*) in *Saccharomyces;*

kynureninase (*175*) and tyrosinase (*176*) in *Neurospora;* adenase (*170*) and uricase (*171*) in *Torulopsis;* cellulase in *Trichoderma* and a basidiomycete (*115*); and α-glucosidase in *Candida* (*180*).

A few attempts have been made to find out whether the appearance of these enzymes is due to enzyme synthesis. First, the appearance of *Saccharomyces* maltase (*162, 179, 181, 181a*), catalase (*178*), cytochrome oxidase (*182*), succinic-cytochrome c-reductase (*182*), and fumarase (*182*) have been shown by indirect devices (amino acid analogs and ultraviolet light) to require protein synthesis. Second, in the case of *Neurospora* tyrosinase, uninduced cells have been shown to lack a protein that reacts with an antibody to highly purified tyrosinase (*176*). Third, the appearance of cytochrome c in *Saccharomyces* in response to oxygen was shown by tracer incorporation studies to result from *de novo* synthesis of the enzyme protein (*183*). On the other hand, the appearance of L-lactic dehydrogenase in this species is not inhibited by amino acid analogs and very probably results from activation of a pre-existing protein (*182*).

In summary, the ability of substrates to elicit the appearance of enzymes is widespread in fungi and the mechanism underlying the processes is sometimes, although not invariably, enzyme induction.

It is worth adding that a case of permease induction has been reported recently in fungi (*184*).

b. Metaphyta. Enzymes can be made to appear by their substrates in metaphytes, i.e., multicellular land plants. All the known examples occur in seed plants (phylum Spermatophyta), both among gymnosperms (*185*) and angiosperms. Angiosperm examples are the most common and are found in both classes, namely dicotyledons (*186–190*) and monocotyledons (*191–194*). As the spermatophytes have evolved furthest anatomically from the algae, one guesses that similar phenomena will be found in anatomically more conservative phyla like the Pteridophyta (including ferns, clubmosses, horsetails, and whisk ferns) and Bryophyta (liverworts and mosses) when they receive some biochemical attention.

According to most of these reports, the enzyme appears so soon after exposure to the inducer (usually within 24 hours) that selection of cells containing the enzyme at the expense of cells lacking the enzyme could hardly account for the results. Again, in most cases, the enzymes were assayed in cell-free systems, thereby avoiding the possibility that permease inductions could explain the results; but in two cases assays were performed only with intact cells (*185, 187*). In another case, only secreted enzyme was measured, raising the possibility that enzyme secretion was induced rather than enzyme formation (*189*).

No evidence has yet been obtained that the appearance of enzyme in response to substrate in seed plants is actually because of induced synthesis of the enzyme. Indeed the first report of "adaptive enzyme formation" in seed plants actually included evidence that the appearance of the enzyme glycolate oxidase in etiolated seedlings treated with glycolate was due to activation of a pre-existing protein (*191*); the appearance of enzyme could be elicited equally well when extracts of untreated etiolated seedlings were incubated with glycolate for 18 hours at 1° C. This seems to have been overlooked by previous reviewers, who have referred to it as a case of enzyme induction.

Likewise, unsuccessful attempts were made to demonstrate that the appearance of nitrate reductase in response to nitrate requires protein synthesis (*190*). Increases in the level of the enzyme were not inhibited by Chloromycetin or the amino acid analogs *p*-fluorophenylalanine or β-thienylalanine, nor by several other antimetabolites and antibiotics. The fact that actidione, DCDNS, and polymixin B did inhibit appearance of the enzyme means little until we know whether any of them inhibit protein synthesis in this system, and if so whether they inhibit protein synthesis specifically, i.e., without inhibiting other parts of metabolism. Since nitrate reductase is unstable inside the cells and is stabilized by nitrate (*190*), one wonders whether this stabilization could account for the appearance of the enzyme when plants are exposed to nitrate.

In conclusion it seems that while there is no doubt that substrates can often bring about the appearance of enzymes in seed plants, there is no evidence yet that the substrates do this by inducing the formation of these enzymes.

c. Metazoa. Several substrates elicit the appearance of enzymes also in multicellular animals. All the known examples come from the phyla that have evolved furthest anatomically from the protista, namely, arthropods and vertebrates. So one might be inclined to guess that the other 15 or 20 metazoan phyla will also be found to exhibit the phenomenon were it not for the fact that the evolutionary position of some of these phyla is rather obscure.

When thyroxine was included in the diet on which insect larvae (*Corcyra cephalonica*) were raised, the level of deiodinating activity exhibited by homogenates was two to ten times higher than when the diet lacked thyroxine (*195*). No data were given to rule out the possibility that thyroxine brought about a selective proliferation either of larvae with the enzyme or of cells with the enzyme. Also, since the deiodinating enzyme was assayed by incubating homogenates of whole larvae for 5 or 16 hours at 37° in the absence of antibiotics, it is possible that the enzyme could have been produced by bacteria from the gut. Finally, no evidence has yet been presented that the appearance of enzyme was due to its synthesis. Nevertheless, this may turn out to be the first example of induction in an invertebrate, and so further results are awaited with interest.

There are several reports of substrate-induced enzyme appearances in vertebrates. Most of these deal, not unexpectedly, with laboratory and domestic strains of a few placental mammals. Some examples of this sort are: tryptophan pyrrolase (formerly known as tryptophan-peroxidase-oxidase) in the rat, guinea pig, rabbit, and dog (*196–203*); xanthine oxidase in the mouse and rat (*204*); threonine dehydrase in the mouse and rat (*205*); benzpyrene hydroxylase in the rat (*206*); tyrosine transaminase in the rat and guinea pig (*207–210*); a steroid dehydrogenase in the mouse (*211*); arginase in the mouse and man (*212, 213*); and alkaline phosphatase in man (*213a*). These species represent three of the four cohorts into which placental mammals are divided (*214*), and so the phenomenon has wide taxonomic distribution. It is of interest from a comparative point of view that thyroxine raises the level of a deiodinating enzyme three to four times in thyroidectomized rats (*215*), since as mentioned above a similar finding was reported for an invertebrate.

Enzymes appear in response to their substrates in two other vertebrate classes, birds and amphibians: acetylcholine esterase (*216*), alkaline phosphatase (*217*), and arginase (*212, 218*) in the domestic fowl and tryptophan pyrrolase in the frog *Rana pipiens* (*219–221*). Adenosine was claimed to induce the appearance of adenosine deaminase in chick embryos (*222*), but more recent studies have questioned the results (*223, 224*). Nevertheless, enzyme adaptation evidently promises to be widespread among vertebrates.

We can now ask whether these phenomena are inductions. In the first place, the substrate might act by inducing certain cells with a high enzyme content to grow faster than other cells in the organism or tissue culture studied. This has usually been ruled out by observing that the enzyme appears too quickly to be accounted for in this way. For example, most of the enzymes referred to above increase and reach their maximum level within 24 hours or less after exposure to the substrate, yet the doubling time of vertebrate cells under optimum conditions is generally thought to be 24 hours or more. But selection might account for the fact that xanthine oxidase takes about 2 weeks to increase by 50% in response to xanthine (*204*). Selection might also account for the threefold rise that takes place in the 11-β-hydroxysteroid dehydrogenase activity of the thymus when mice receive cortisol (an 11-β-hydroxysteroid) for 9 days, as the histological appearance of the thymus changes during this time (*211*).

In the second place, the enzymes dealt with have generally been assayed in cell-free systems, thereby avoiding the possibility that permeases account for the results.

However, only a few indirect attempts have been made to see whether the substrate-induced appearance of enzymes in vertebrates is due to enzyme synthesis. The methionine analog ethionine has been used in a number of cases. It inhibits permease induction in some bacteria without inhibiting their respiration (*126*) and it inhibits maltase induction in yeast (*179*); but as far as is known, this analog is not a specific inhibitor of protein synthesis. It is known to be incorporated into protein, in place of methionine, during protein synthesis (*225–227*), but it remains to be ascertained whether the resulting altered proteins retain their biological activity. The only such protein so far studied, a crystalline amylase from *Bacillus subtilis* (*227*), is physicochemically and enzymatically identical with the natural enzyme even though all four of the methionine loci are occupied partly by ethionine. Since methionine functions in metabolism not only as a constituent of proteins, but also, for example, as a participant in methyl group metabolism (*81*), it might be best to be cautious about interpreting ethionine's inhibitory effect on substrate-induced appearances of enzymes as evidence that protein synthesis is required.

In such complex organisms as vertebrates it is conceivable that some metabolic processes could be interfered with by agents that inhibit protein synthesis or cause altered proteins to be made even though these processes might not themselves directly involve protein synthesis. This possibility could be avoided to some extent by working with simpler systems than whole animals, as was done in the case of threonine dehydrase (*205*) and tryptophan pyrrolase (*200*), whose appearance can be elicited in isolated perfused liver. As well as being dependent on the presence of substrate, the appearance of enzyme is dependent in each case on there being a complete mixture of amino acids in the perfusate, and it is inhibited by ethionine. Hence, the appearance of enzyme in this system still seems to require protein synthesis. However, it should be pointed out that in the threonine dehydrase experiment ethionine also inhibited the production of bile very strongly, a process that could scarcely be expected to require protein synthesis. Consequently, ethionine's effect on the appearance of enzyme might not be due to inhibition of protein synthesis.

Even simpler systems have been used. For example, substrates have been reported to induce the appearance of enzymes in tissue cultures containing a single cell type (*213, 213a*), in dissociated embryonic cells (*219, 220*), and in tissue or organ fragments taken freshly from embryos (*212, 216, 217*). No experiments have yet been done to see whether protein synthesis is required for the appearance of enzyme in these systems.

Attempts have been made to use more direct methods to determine whether substrate-induced enzyme appearance in mammals is due to enzyme synthesis. Gros *et al.* (*197*) showed that when rats or rabbits were treated with C^{14}-valine as well as the substrate tryptophan, a liver protein fraction containing tryptophan pyrrolase achieved a specific activity a few times higher than another protein fraction synthesized mainly by the liver, namely, serum proteins. In control animals which received no tryptophan the liver protein fraction achieved a specific activity that was slightly lower than that of the serum proteins. Hence, tryptophan selectively stimulates synthesis of the protein fraction containing tryptophan pyrrolase. It might be inferred from this that the synthesis of the tryptophan pyrrolase protein was stimulated; but, as the protein fraction was impure, proof would require measurements of the specific activity of the pure protein. Indeed, the most recent evidence suggests that the appearance of tryptophan pyrrolase in response to tryptophan is largely due to activation of a pre-existing protein (*228–230*).

Another type of direct approach has been made with tyrosine transaminase, which appears in rat liver following injection of tyrosine or glucocorticoids (*231*). The enzyme was purified more than 500-fold from rat livers and used as an antigen. The antigen-antibody reaction studied by the double-diffusion technique suggested that there was a single antigen. Then, when the antibody was used to measure the amount of antigen present in liver extracts, it was revealed that "induced" extracts contained no more antigen than extracts of normal rat livers, despite the fact that the induced extracts exhibited more than thirty times as much transaminase activity. This appeared to be strong evidence against enzyme induction being the mechanism underlying the appearance of transaminase activity until it was learned very recently (Kenney, personal communication) that the method used to measure the amount of antigen in the liver extracts had been faulty.

A very similar experiment was done with ferritin, an iron-binding protein, which appears in the liver when iron is injected. Crystalline ferritin was used to generate a specific antibody which was in turn used for measuring the amount of antigen present in extracts of livers from control and iron-injected guinea pigs. The results clearly showed that iron acts by inducing the formation of the protein part of ferritin (*232*).

Similar findings have since been made with cultured HeLa cells (*233*).

In conclusion, there are now a variety of cases in which a substrate has been found to elicit the appearance of an enzyme in metazoa. Some of these cases, like that of tryptophan pyrrolase, are almost certainly not due to induction of the synthesis of the enzyme. On the other hand, there is no doubt that mammals, at least, do have the capacity to synthesize certain proteins, such as ferritin, selectively. Yet it remains to be proved that a substrate can elicit the appearance of an enzyme by inducing its synthesis in metazoa, or in any multicellular organisms for that matter.

C. ENZYME REPRESSION

1. *Enzyme Repression in Bacteria*

Another type of control mechanism, called repression, was discovered in the biosynthetic pathways of coliform bacteria. Repression is the specific inhibition of the synthesis of an enzyme by a more or less distant product of the reaction catalyzed by the enzyme. As will be seen later, its mechanism is basically the same as that of induction.

An early indication that repression occurs came from an experiment with tryptophan synthetase (tryptophan desmolase), an enzyme that condenses indole and serine to tryptophan. The activity of this enzyme, as measured in washed cells or in dialyzed extracts of *Aerobacter aerogenes,* was 70% reduced if instead of being grown in minimal medium the bacteria were grown overnight on minimal medium plus tryptophan (*234*). Tryptophan did not affect the stability of the enzyme in extracts. Also, mixtures of extracts from cells grown with and without tryptophan exhibited an activity that was the sum of the separate activities measured before mixing. Hence, there was probably no inhibitor or activator in the extracts. This warranted the hypothesis that tryptophan inhibits the synthesis of an enzyme in its own biosynthetic pathway. The effect was chemically specific in that only some substituted tryptophans or indole (which could give rise intracellularly to tryptophan) could mimic the effect of tryptophan; other amino acids did not affect the synthesis of the enzyme much.

Similar specific effects on the methionine synthetase activity of washed *E. coli* suspensions were noted when the medium contained methionine during growth (*234, 235*). A further indication of the generality of the phenomenon was the finding that the inclusion of valine in minimal medium during growth reduced the level of an enzyme of valine biosynthesis in *E. coli* (*236*). Later, similar phenomena were reported in the biosynthetic pathways of arginine (*237–240*), pyrimidines (*241*), histidine (*20*), tyrosine (*242*), cysteine (*243*), purines (*244*), and DPN (*245*). Further investigations have been made of repression in the biosynthetic pathways of tryptophan (*246, 247*), methionine (*248, 248a*), and valine (*9, 249*) [for reviews, see (*250, 251*)].

Although all the examples of repression referred to so far occur in the biosynthetic pathways of coliform bacteria (*Escherichia, Aerobacter, Salmonella, Serratia,* and *Proteus*), there is also evidence that early enzymes of porphyrin synthesis may be repressible in *Rhodopseudomonas spheroides* (*252*).

Repression does not occur only in biosynthetic pathways. Alkaline phosphatase is repressed in 3 out of 10 strains of *E. coli* by inorganic phosphate, a product of the reaction catalyzed by this enzyme (*41, 253, 254*). The activity of the enzyme rises when these strains are allowed to grow in the absence of exogenous inorganic phosphate. Consequently this aspect of the phenomenon is designated derepression. The phenomenon also occurs in *Bacillus subtilis,* but not in any of 20 strains of *Staphylococcus aureus* (*254, 255*). The inducible tartrate dehydrases of a *Pseudomonas* species are repressible by oxalacetate, the product of the reactions they catalyze (*256*). In addition, isocitratase, a key enzyme of the glyoxylate cycle is repressible by succinate in *Micrococcus denitrificans* (*66*).

The possibility that selection of mutants might account for changes in enzyme levels described as repression has been ruled out in several reports. For example, when pyrimidineless *E. coli* mutants growing in medium containing a pyrimidine are suddenly shifted to a medium that differs only in lacking the pyrimidine, the rate of formation of the first enzyme in the pyrimidine pathway, aspartate transcarbamylase (ATCase) rises by a factor of about a thousand within a few minutes (*241*). Similar results have been obtained from derepression experiments with alkaline phosphatase (*41, 253*) and some enzymes of purine synthesis (*244*).

Pyrimidine synthesis has provided the most critical evidence to date that actual changes in the amount of enzyme protein are responsible for the changes in enzyme activity. The increase in ATCase activity that takes place when pyrimidines are withheld from a pyrimidineless mutant is prevented by inhibitors of protein and enzyme synthesis such as chloramphenicol, 5-methyltryptophan, and ultraviolet light. Furthermore, when the mutants are deprived of pyrimidines in the presence of leucine-C^{14} and then extracts made from them are fractionated electrophoretically, the specific radioactivity of the protein is high enough in the fraction that contains the ATCase activity to account fully for the observed increase in the enzyme's activity relative to other enzymes (*241*). Similar results using proline-C^{14} incorporation and DEAE-cellulose fractionation showed that derepression of *E. coli* alkaline phosphatase is due to *de novo* protein synthesis (*42*).

The effect of a biosynthetic end product on the synthesis of enzymes in its own pathway might be explained in two ways. The end product might inhibit the activity of an early enzyme in the pathway (for example by feedback inhibition, see Section II, A), thereby reducing the intracellular concentration of intermediates in the pathway. If biosynthetic intermediates act as inducers of the enzymes for which they are substrates, the rate of synthesis of these enzymes would in turn be reduced. Another explanation would be that the end product directly inhibits the synthesis of these enzymes.

The experiments of Vogel (*237*) and Yates and Pardee (*241*) allowed a choice to be made between these two explanations. For example, several mutants unable to synthesize intermediates early in the pyrimidine pathway contained the same low level of early enzymes in this pathway when grown on a pyrimidine as wild-type bacteria, but contained large amounts of the enzyme when starved of pyrimidines. However, the mutant cells would lack some early intermediates under all conditions since an early step of the pyrimidine pathway is missing and the pathway is irreversible at a step beyond its mid-point. The inhibition of enzyme synthesis by a pyrimidine is therefore direct. Analogous results were obtained for the arginine pathway (*237*) and later for the histidine pathway (*20*).

More recently Gorini (*240*) has shown that ornithine, an intermediate in arginine biosynthesis, can induce the formation of the enzyme for which it is a substrate. It does this by interfering with the repression exerted by arginine. However, ornithine is not needed to enhance the synthesis of the enzyme, because when arginineless mutants are deprived of arginine the enzyme becomes derepressed whether or not ornithine is present in the cells. This finding suggests that, as well as inhibiting directly, biosynthetic end products can influence the formation of enzyme in their pathway indirectly by feedback inhibition.

Induction of β-galactosidase (and probably other enzymes) also involves repressors. Galactosides induce the enzyme by interfering with its repression by an intracellularly formed repressor (*257*). The parallel goes further because, as well as mutations that make an inducible system constitutive, there are mutations that make repressible systems non-repressible. For example, there are mutants in which tryptophan cannot repress the enzymes of tryptophan synthesis (*246*). These mutations occur in a genetic locus (regulator locus) distinct from the linked loci that determine the structure of these enzymes. This suggests that tryptophan itself is not the repressor but acts in conjunction with a substance produced by the repressor locus. Interestingly enough, a single mutation in the repressor locus abolishes all at once the ability of tryptophan to repress several of the tryptophan biosynthetic enzymes, just as a mutation in the *i* gene of the galactosidase system affects the inducibility of both galactosidase and galactoside permease. As a result of mutations in the repressor locus, tryptophan's biosynthetic enzymes are synthesized at a high rate under all growth conditions, i.e., constitutively.

Mutations conferring constitutivity have now been observed also in the biosynthetic pathways of pyrimidines (*16*) and arginine (*257a*) and in the alkaline phosphatase system (*258, 259*).

2. *Repression in Other Organisms*

a. Algae, Protozoa, Fungi, and Metaphyta. No instances of repression are known among protozoa or algae. Possibly there are several instances in seed plants and fungi, two of them in biosynthetic pathways. Proline seems to repress pyrroline-5-carboxylate reductase, an enzyme of proline synthesis, when *Neurospora* grows on minimal medium supplemented with proline, but the effect is slight (*260*). Tryptophan synthetase and some earlier enzymes in tryptophan's biosynthetic pathway are also repressible in *Neurospora.* When a mutant blocked before anthranilic acid is grown with a low concentration of tryptophan, the level of tryptophan synthetase is about three times higher than in the wild-type organism, but the enzyme is not repressed below the wild-type level when growth takes place in high concentrations of tryptophan (*18*). Earlier enzymes in the pathway, however, are repressed strongly when a mutant lacking tryptophan synthetase is grown in excess tryptophan (*18*).

The proline and tryptophan experiments were short enough in comparison with the generation time of *Neurospora* to make it unlikely that selection of mutants could account for the results. However, no evidence was offered that the changes in enzyme activity observed were due to changes in amounts of enzyme proteins.

Some other amino acids, for example, histidine (*20*) and valine and isoleucine (*261*), do not repress enzymes in their biosynthetic pathways when added at high concentrations to *Neurospora* in minimal growth medium. However, these enzymes might be derepressed under conditions in which the intracellular concentration of the amino acids was forced to be abnormally low.

A phosphatase is apparently repressible by inorganic phosphate in the yeasts *Torulopsis* (*43*) and *Saccharomyces* (*262*), as the specific activity of this enzyme in cell-free preparations (dialyzed or undialyzed) rises about tenfold during a few hours of growth in a low-phosphate medium. But when *Neurospora* is grown in a low-phosphate medium, the specific activity of alkaline phosphatase does not rise (*254*).

Isocitratase and malate synthetase are probably repressible by succinate in *Saccharomyces drosophilarum* (*263*).

Finally, there is a report that *Neurospora* tyrosinase is repressible by sulfate (*176*). The function of this repression is not yet understood. This report is, nevertheless, of considerable interest from a comparative point of view because the authors show that besides a genetic locus that determines the structure of the enzyme there are loci that affect the inducibility and repressibility of the enzyme. This implies that induction and repression in fungi have mechanisms like those found in bacteria.

Besides reports of repression in fungi there is preliminary evidence that glutamine represses glutamyl transferase in a seed plant (*263a*) and there is similar evidence for an animal (see below).

b. Metazoa. There is evidence that repression may occur both in vertebrates and in invertebrates. The best example with an invertebrate concerns the hemoglobin of small mollusks and arthropods that live in ponds, e.g., certain young pond snails (*Planorbis*), certain midge larvae (*Anatopynia* and *Chironomus*), and a variety of crustaceans, including anostracans, notostracans, cladocerans, and conchostracans. When the water is well aerated these organisms contain little hemoglobin, but when it is poorly aerated they gradually become pink owing to enhanced synthesis of hemoglobin (*264*). When the water flea (*Daphnia*), for example, is transferred from well to poorly aerated water, the hemoglobin concentration builds up quickly, reaching five times the starting level after 5 days and a maximum of ten times the starting level by 2 weeks (*265*). It has also been reported briefly that cytochromes are synthesized by *Daphnia* in response to oxygen lack (*264*). By raising their hemoglobin content, water fleas and brine shrimp (*Artemia*) greatly improve their chances of surviving in poorly aerated water (*266, 267*).

The experiments of Fox and co-workers suggest that oxygen represses hemoglobin synthesis in these invertebrates (*264, 265, 268*). The rapidity with which hemoglobin appears when the oxygen concentration falls would seem to rule out a selective proliferation of hemoglobin-synthesizing cells as a cause. However, it should be noted that the slight rise in hemoglobin content that takes place when mammals are exposed to low oxygen pressures is due to selective proliferation of such cells, i.e., hematopoiesis (*269*). Another possibility is that oxygen acts by inhibiting heme synthesis (*270*) without affecting globin synthesis.

The effect of oxygen on the appearance of hemoproteins is widespread but not universal. No differences in hemoglobin content were detected when groups of individuals of the following species were made to live and grow for several weeks at markedly different oxygen concentrations: one rhabdocoel flatworm, two young polychete worms, one young oligochete worm, and two adult snails (*264*). However, several bacteria raise their cytochrome content in response to low oxygen tension (*271*).

We know of only one other possible instance of repression in an invertebrate. This concerns the fructose-specific site on the chemoreceptor neuron in the sensory hair of the blowfly leg. The development of this stereospecific site, presumably a specific protein, is inhibited if the larva develops in a medium containing fructose (*272*).

The best example of repression in a vertebrate concerns glycine transamidinase, the first enzyme in the biosynthetic pathway of creatine:

Glycine → Guanidoacetate → Creatine

When chicks, for example, are shifted from a diet lacking creatine to one containing 2% creatine, the level of this liver enzyme, measured in homogenates, begins to fall with a half-life of about 20 hours; after a few days it may be fifty times lower than the original level (*273, 274*). The levels of some other liver enzymes, e.g., the second enzyme in the pathway (guanidoacetate methylferase) and arginase, do not change under these circumstances (*273*). The depressed level of transamidinase is not restored by overnight dialysis, treatment with trypsin, or incubation with cysteine; and when liver homogenates from creatine-fed chicks are mixed with control homogenates the activities are additive (*273*). Moreover, neither creatine nor creatine phosphate inhibits the activity of the enzyme (*273, 275, 276*). This implies that transamidinase activity changes because of changes in enzyme amount.

When creatine-fed chicks are shifted back to the control diet, the transamidinase activity rises quickly and reaches the original value in a few days. The activity does not return if the chicks are fasted. However, a diet containing only carbohydrate and casein hydrolyzate plus tryptophan suffices to restore activity. If the tryptophan is left out, the diet is ineffective. This suggests that protein synthesis is needed for the restoration of transamidinase activity. In support of this, ethionine inhibits the restoration (*274*). Although direct evidence that the restoration represents *de novo* synthesis of the enzyme is still lacking, a likely explanation of the results obtained so far is that creatine represses the synthesis of transamidinase.

Transamidinase is also apparently repressible in ducklings and rats

(*274–276*). The phenomenon may therefore be common in vertebrates. This is understandable in view of the evidence that it is of considerable adaptive significance (*273, 274*). In the first place, repression of the enzyme is effective in slowing down the pathway of creatine synthesis, i.e., in the repressed state transamidinase is rate limiting. In the second place, dietary creatine has been shown to spare a portion of the arginine requirement of chicks on artificial diets. (Arginine is the source of the amidine group transferred to glycine.) Third, some natural food sources such as vertebrate muscle contain a high enough proportion of creatine (0.5%) to cause a marked repression of transamidinase. Animals like ducks and rats, which include vertebrate muscle in their natural diet, would therefore have a selective advantage over species with similar diets but a nonrepressible enzyme.

Before finishing this discussion of transamidinase repression it is worth noting a comparative aspect of the problem: guanidoacetate represses the enzyme as strongly as does creatine in the chick (*274*). This is probably not a direct inhibition but a result of guanidoacetate conversion to creatine in the liver of the chick. This conclusion arises from a comparison with the rat, in which transamidinase occurs in the kidney, i.e., separately from the methylferase in the liver, and in which transamidinase is not repressible by guanidoacetate (*275*). This illustrates how a comparative biochemical approach can give clues about the mechanisms underlying metabolic control processes.

Repression might occur in some other biosynthetic pathways of vertebrates. It has been known for some time that cholesterol somehow inhibits its own synthesis [for reviews see (*277, 278*)]. If the cholesterol content of the diet of the rat is raised, the rate of cholesterol synthesis from C^{14}-acetate begins to fall and within a few days reaches a minimal value that is one or two orders of magnitude lower than the starting value. When cholesterol is removed from the diet the rate of cholesterol synthesis returns to normal over the course of a few days. These results have been obtained by measuring cholesterol synthesis *in vivo*, in tissue slices, and in homogenates from which the mitochondria were removed by centrifugation. The cholesterol effect is especially pronounced early in the pathway because cholesterol synthesis from C^{14}-mevalonate is not inhibited by cholesterol feeding nearly as strongly as from C^{14}-acetate (*279–281*). Cholesterol has no effect on cholesterol synthesis when added to liver slices (*281*). Therefore a possible explanation of the effect is that cholesterol inhibits the formation of enzymes in its biosynthetic pathway. But there is not yet any evidence that protein synthesis is required for the restoration of the ability to synthesize cholesterol.

There are indications that the cholesterol effect occurs in a considerable variety of laboratory animals, both birds and mammals (282), although in some species the effect is quite small and takes weeks to be expressed. In such cases repression might not be involved, but rather selection of cells.

Repression-like effects occur in the pyrimidine biosynthetic pathway of man. As mentioned earlier, the inhibitory effect of pyrimidines on orotic acid excretion by a child afflicted with orotic aciduria (25) could be attributed to feedback inhibition of the activity or the formation of the orotic acid-synthesizing enzymes. The following evidence suggests that the latter possibility is more likely. The blood cells of people with pernicious anemia, in which pyrimidine synthesis apparently takes place at a subnormal rate, have an elevated level of aspartate transcarbamylase and dihydro-orotase (283). Furthermore, vitamin B_{12} therapy brings about not only a remission of the hematological symptoms of the disease and a stimulation of pyrimidine synthesis, but also a return of the levels of the two enzymes to normal (283).

Relatives of the orotic-aciduric child (now deceased) have recently been found to possess normal levels of early enzymes of the pyrimidine biosynthetic pathway; but in both parents and in two of the three surviving children, two enzymes active later in the pathway, orotidylate pyrophosphorylase and orotidylate decarboxylase, are found at reduced levels, roughly three to five times lower than normal. Probably, then, orotic aciduria is an inborn error of metabolism, occurring in individuals homozygous for the trait that makes heterozygotes have low levels of these two enzymes (*283a*).

However, inborn errors of metabolism in man are usually single enzyme defects resulting from mutation of a single structural gene. We may speculate then that in the present case the inborn error is a mutation in a gene which regulates the synthesis of the two enzymes. If so, this would be the first instance of a regulator gene in a multicellular organism and would suggest that repression-like phenomena in multicellular organisms can have mechanisms similar to those in bacteria.

Purines are believed to repress early enzymes of their own pathway in mouse fibrocytes (284); but the evidence, which comes from isotopic competition experiments with cells growing *in vitro*, is still incomplete.

A preliminary communication presented evidence that glutamyl transferase of HeLa cells (in tissue culture) appears to be repressed by glutamine (285). A fifteenfold difference in enzyme level was observed between fully repressed and fully derepressed cells; evidence was given that glutamine does not act by selecting mutants.

Glutamate prevents the rise in level of kidney glutaminase I that takes place when guinea pigs are treated with acidifying agents (286).

As the enzyme's appearance is prevented also by ethionine, this may be an instance of repression. Glutamate is reported also to lower the level of glutaminase I in the retina of rats and mice, but no experimental details are yet available (*287*).

Alkaline phosphatase, as mentioned earlier, is repressible by inorganic phosphate in certain bacteria. The enzyme is not de-repressed when cultured cell lines from human skin grow in a low-phosphate medium, nor is the marked rise in enzyme level that occurs in response to substrates or glucocorticoids inhibited by high concentrations of phosphate (*213a, 287a*).

Finally, it was claimed that when large amounts of tyrosine were fed to rats the phenylalanine hydroxylase activity of liver fell threefold in 5 hours and reached a minimal value roughly thirtyfold below normal (*288, 289*). Also, radioactive tyrosine appeared in the proteins of a certain strain of human cells incubated with radioactive phenylalanine only if tyrosine was absent from the medium (*290*). Yet tyrosine does not inhibit the activity of phenylalanine hydroxylase (*291*). The evidence therefore implies that tyrosine represses the enzyme. However, more recent work shows that tyrosine does not reduce the level of this enzyme in rat liver; the earlier reports to the contrary resulted from a faulty method of assaying the enzyme (*292*).

In summary, repression-like mechanisms of controlling metabolism occur in a variety of eubacteria, in fungi and seed plants, and in a variety of anatomically complex animals (mollusks, arthropods, and vertebrates). True repression, the inhibition of enzyme formation, is the mechanism in at least some of these bacteria and fungi; but it remains to be ascertained by direct methods, i.e., immunochemical and tracer-incorporation methods, that the repression-like effects in other organisms result from changes in rates of enzyme synthesis.

IV. Activation of Enzymes

Changes of enzyme levels in response to exogenous substrates or products (direct or eventual) are by no means always caused by changes in rates of enzyme formation. Some other mechanisms for controlling enzyme amount can be loosely grouped together as activations. Examples have been noted in previous sections and further examples will be presented in this section. They support the suggestion that besides induction and repression there are other, quite general, mechanisms for controlling enzyme levels.

Substrates can activate a pre-existing protein and thereby give rise to a phenomenon that resembles induction. Examples are available from many phyla. We have already noted the case of *E. coli* DNase (*83*).

Another bacterial example is the activation of hydrogenase by hydrogen in *Proteus vulgaris* (*293, 294*). Interestingly enough, the appearance of hydrogenase in *Hydrogenomonas facilis* may be due to enzyme synthesis, as chloramphenicol inhibits the process (*91*). Hydrogenase is, as we saw, also activated by hydrogen in certain algae (*139*). In the slime mold *Dictyostelium,* substrates may raise the level of isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase by a process of activation (*295*). Other previously mentioned examples include L-lactic dehydrogenase in true fungi (*182*) and glycolate oxidase in seed plants (*191*). Some examples of substrate activation also occur in mammals. Tryptophan pyrrolase has already been mentioned (*228–230*). Another good example is thymidylate kinase, whose level, as measured in liver homogenates, goes up severalfold within a few hours after injection of thymidine (*296*); evidence was obtained that the thymidine acts by being converted to thymidylate. However, the rise in enzyme activity can be accounted for by the fact that thymidylate activates the enzyme (*297*). In sum, the ability of substrates to elicit the appearance of enzymes by activation of pre-existing proteins, rather than by induction of enzyme synthesis, is widely distributed in the living world.

Some of the reported instances of induction that have not been studied thoroughly will undoubtedly turn out to be activations. Recent discoveries about the mechanism underlying the appearance of tryptophan pyrrolase activity in response to tryptophan bear out this assertion in a telling way. Although tryptophan pyrrolase was the most thoroughly studied metazoan example of an apparently inducible enzyme, its appearance has been shown now to be mainly the result of an activation process (*228–230*).

Superficially repression-like effects can be caused by activations (or inactivations). When a strain of mouse fibrocytes is grown in the presence of products (or the substrate) of the reaction catalyzed by DPNase, the DPNase activity is much lower than that of cells grown in the absence of such compounds. These compounds exert their effect by irreversibly inactivating the enzyme (*298*). This has incorrectly been referred to elsewhere as an instance of repression.

Many other enzymes can be synthesized in an inactive form. Some examples are the vertebrate proteinases (*299*); phosphorylase in vertebrates and arthropods (*300*); tyrosinase in insects (*301–303*) and fungi (*304*); glucose-6-phosphate dehydrogenase in man (*305, 306*); *p*-hydroxyphenylpyruvate oxidase in guinea pigs (*210*); arginase in chicks (*307*); succinoxidase in mammals (*308*); DNase in mammals, yeast, and bacteria (*309*); DPNase (*310*), a proteinase (*311*), an amylase (*312*) and glycerol dehydrogenase (*117*) in bacteria; and indoleacetic acid oxidase in seed plants (*313*).

The mechanisms underlying what we have termed activations are diverse and not yet well characterized. In some cases the inactive enzyme may be in the form of a typical zymogen, which often has to be activated by a second enzyme. This type of mechanism is responsible for activation of chymotrypsin (*314*), carboxypeptidase (*315*), plasmin (*316*), fructose diphosphatase (*317*), phosphorylase (*300*), and glucose-6-phosphate dehydrogenase (*306*). The activating enzyme is often proteolytic; but in the case of phosphorylase the activating enzyme is a kinase.

It can be noted in passing that as zymogens can differ immunochemically from the activated form of the enzyme (*318, 319*), immunochemical methods are not always sufficient by themselves to distinguish between activation and *de novo* synthesis of enzymes.

Instead of being in the form of zymogens, some enzymes are inactive because of the presence of tightly bound specific inhibitors. These inhibitors are generally large molecules—proteins in some cases (*308, 310, 320, 321*), ribonucleic acids in others (*83, 307*). Cells may also contain activators that oppose the action of some of these inhibitors (*321–323*).

In still other cases, enzyme levels may vary in cells because of variations in the degree to which an enzyme is protected from inactivation or destruction (*117, 295, 297*).

V. Discussion

The preceding survey will have made it evident that control mechanisms affecting enzyme activity (the rate of functioning of enzyme molecules) and enzyme amount (the number of functional molecules of enzyme) are widespread in the living world. Possibly they occur in all phyla. This is understandable because such control mechanisms appear to be of selective value to all cells; for the control mechanisms we have discussed appear to benefit cells by making metabolism more efficient or economical.

Thus, feedbacks affecting enzyme activity enable cells (*a*) to avoid overproducing metabolites, (*b*) to stimulate lagging metabolic pathways, and (*c*) to allow exogenous metabolites to be used in preference to synthesizing them (*236, 251, 324, 324a*). Control mechanisms affecting enzyme amount also function in this way (*325*), although less efficiently because they act more slowly (*324, 324a*).

As we have seen, there are two general sorts of mechanisms for controlling enzyme amount: (*a*) mechanisms affecting enzyme formation (induction and repression) and (*b*) various other mechanisms (activations). The mechanisms affecting enzyme formation improve the efficiency of metabolism by helping both to effect an economy in the production of small molecules (metabolites) and also to effect an economy in protein synthesis (*324, 325*). Thus when *E. coli* grows in a medium containing a β-galactoside, more than 5% of the proteins synthesized are β-galactosidase, but as soon as the inducer is removed galactosidase constitutes less than 0.05% of the proteins synthesized (*69*). And *Pseudomonas fluorescens,* which has been estimated to contain at least 200 inducible enzymes in its repertoire (*88*), must often encounter environmental situations where most or all of them would be useless. These examples illustrate that the saving in energy and materials effected by controls over enzyme formation can be considerable. This would appear to give such control mechanisms an advantage over activation mechanisms.

It remains then to be explained why activation mechanisms seem so widely distributed in the living world. Because of their speed these mechanisms might sometimes be superior to induction-repression in controlling the metabolism of small molecules. However, activation is not always faster than induction-repression. Thus in the case of mammalian liver, the kinetics of appearance of tryptophan pyrrolase (*202*), and thymidylate kinase (*296*) as a result of activation are rather similar to the kinetics of appearance of ferritin (*326*) as a result of *de novo* protein synthesis. Clearly more work is needed to explain the adaptive value of these cases of activation.

Although mechanisms for controlling enzyme activity and enzyme amount are evidently widespread, too little is known about the details of the mechanisms and about their distribution to warrant drawing many conclusions of a comparative biochemical nature. However, the survey does indicate possible differences between these mechanisms in different organisms. For instance, in adult multicellular organisms, activations may be a more common mechanism of adjusting enzyme levels than in bacteria and fungi, which commonly use repression and induction for these purposes. This may be connected with the fact that the rate of protein synthesis in adult multicellular organisms is comparatively low.

Special ecological groups may have unusual control mechanisms. The algae and fungi that live symbiotically within the cells of invertebrates might have weak feedback control mechanisms, which would enable metabolites to be overproduced for the host cells. Other possible cases of weakened feedbacks may be provided by the bacteria and fungi that excrete antibiotics, especially the strains selected for producing antibiotics on a commercial scale.

The control mechanisms of different cell types in the same organism can be compared. It is already known that enzymes whose appearance can be elicited by their substrates in one organ, e.g., the liver, are not induced to appear in other organs.

Other types of control mechanisms than any we have discussed probably exist. Possibly some of them underlie the slow rhythms of metabolic activity that go on in a wide variety of organisms, even single-celled organisms, when kept under apparently constant environmental conditions (*327*).

As well as helping simply to make metabolism of cells more efficient, control mechanisms may play an important part in more complex biological processes. Considerable interest exists, for example, in the possibility that induction and repression-like mechanisms may control the changing enzyme levels characteristic of such diverse processes as the embryonic development of multicellular animals (*328*), the life cycle of slime molds (*328*) and fungi (*329*), the germination of seeds (*330, 331*), the metamorphosis of larvae (*332–334*), and the birth of placental mammals (*201, 335*), as well as during liver regeneration in mammals (*336, 337*), diapause in insects (*338*), estivation in snails (*339*), dietary changes (*340, 341*), disease (*337, 342*), virus infection (*343–345*), and exposure to drugs and poisons (*346*) and hormones (*198, 341, 347–350*).

Some of these changes in enzyme level do appear to be due to changes in rates of enzyme formation. A notable example is carbamyl phosphate synthetase, an enzyme of urea, arginine, and pyrimidine synthesis, which appears in the liver at the onset of the metamorphosis of tadpole to frog—a time when the organism switches from ammonia excretion to urea excretion. Immunochemical and tracer-incorporation methods suggested that the appearance of the enzyme was the result of *de novo* enzyme synthesis (*351*). However, there are several other cases where such changes are apparently not due to changes in rates of enzyme formation (*201, 303, 317, 341, 352*). Further discussions of the role of induction and repression in development and other complex processes can be found in recent reviews (*328, 353, 354*).

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Note Added in Page Proof

Since the galley proofs were received (December, 1961), several advances have occurred, some of which we have recently summarized [A. B. Pardee and A. C. Wilson, *Cancer Research*, in press (1963)]. Not referred to in that summary, but of particular comparative biochemical interest, is the current survey of feedback inhibition of aspartate transcarbamylase in different taxonomic groups [J. Neumann and M. E. Jones, *Nature* **195**, 709–710 (1962); J. Neumann and M. E. Jones, in preparation; W. D. Donachie, *Biochim. et Biophys. Acta,* in press (1963)].

CHAPTER 3

The Biochemical Basis of Chemical Needs

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I. Introduction 120
  A. Contemporary Status of Comparative Nutrition 120
  B. Biochemical Function as the Basis of Nutritional Requirements . . 122
  C. Nutritional Requirements as a Function of Genetic Capabilities . . 123
  D. Quantitative Evaluation of Chemical Needs 125
  E. The Nutritional Economy of the Biosphere 126
II. The Abiogenic Fulfillment of Biopoietic Demands 128
  A. Origins of Terrestrial Life 128
  B. The Primitive Biosphere 129
  C. Formation of Simple Organic Molecules 130
  D. Formation of Complex Organic Molecules 132
  E. Nutrition on Extraterrestrial Bodies 133
III. Requirements for Maintenance of the Fluid Medium 135
  A. General Considerations 135
  B. The Plant Kingdom 136
  C. The Animal Kingdom 138
IV. The Nutritional Source of Free Energy 141
  A. Photosynthetic Autotrophs 141
  B. Chemosynthetic Autotrophs 143
  C. Heterotrophic Nutrition 146
V. Nutritional Requirements for Structural Organization 148
  A. Proteins 149
  B. Polysaccharides and Lipids 161
  C. Polynucleotides 164
  D. Mineral Deposits 167
VI. Requirements for the Catalysis of Organic Transformations . . . 169
  A. Basic Concepts 169
  B. The Trace Elements 173
  C. Vitamins Involved in Electron Transfer Systems 187
  D. Vitamins Involved in CO_2 Formation and Fixation 195
  E. Vitamins That Labilize α-Carbon Atoms 197
  F. Vitamins That Transfer Acyl Groups 198
  G. Vitamins That Transfer Single-Carbon Units 199
  H. Carotenoid Vitamins 205

I. Steroid Vitamins 208
J. Vitamins with Uncertain Functions 210
VII. Summary and Conclusions 211
References 213

I. Introduction

A. Contemporary Status of Comparative Nutrition

The chemical needs have been more or less defined for about two hundred animal and three hundred plant species. These represent about 0.4% of the estimated number of kinds of animals and plants. In many instances the information is sketchy and has been derived by exceedingly indirect means. The preponderance of the data applies to a very few groups: to the Protozoa, Arthropoda, and Chordata in the animal kingdom and to the Algae, Fungi, and seed plants in the plant kingdom. Virtually nothing is known concerning the nutrition of species in the other numerous and biologically significant phyla and divisions. This situation has developed because nutritional studies are generally motivated by immediate and practical considerations, but seldom as a long-range project or in the basic search for knowledge. Thus, groups have been studied because they are pathogens (protozoa, worms, insects, bacteria, fungi); because they deteriorate lifeless materials of value (insects, bacteria, fungi); because they are food or drug sources (vertebrates, algae, bacteria, fungi, seed plants); because they are important in manufacturing (e.g., the fermentation and silk industries); and because they aid in the understanding of growth, development, general health, and the amelioration of systemic disease.

Other inducements, however, should stimulate a major effort toward the extension of our understanding of comparative nutrition, not the least of which is the search for knowledge for its own sake. Nutritional factors are major but sadly neglected aspects of both plant and animal ecology. The chemical needs of any species are inextricably linked with its biochemistry, physiology, and anatomy, and their definition for that species would seem to be fundamental to studies in these areas of biology. Current concepts of genetics, which have been derived in great part from nutritional parameters (in such genera as *Neurospora* and *Drosophila,* for example) can most expediently be extended by the similar study of the many, diverse, uninvestigated groups. Studies of morphogenesis and differentiation are severely retarded by the *absence* of knowledge regarding the admittedly critical influence of nutritional factors, and most particularly in such valuable animals as hydras, sea urchins, and frogs. Nutritional studies have provided a valuable key to several complex taxonomical problems in the past and may well assist in unraveling the many anomalous situations that still remain. Much remains unknown regarding the function of many of the recognized nutrients, and the study of the requirements for these substances in uninvestigated species may well aid in the elucidation of their role in biochemical systems.

Comparative nutrition appears to provide a most expedient approach to the extension of our knowledge of nutrition itself. The plethora of "unidentified factors" that are reported annually, as well as the rarity of completely chemically defined diets, attests to the existence of numerous unrecognized chemical requirements. Just as bacteria, fungi, protozoa, and some lower vertebrates have already been the means of discovery of many of the presently recognized nutrients, unstudied species may provide the key in future investigations. This is most particularly true when, through some peculiarity of the requirement in a given species, the nature of the substance and of its function is sufficiently exaggerated to simplify study and measurement in that system.

As against the inducements to the study of comparative nutrition, there are many vexing problems in this research area. Availability of many species is limited. Culture methods have been worked out for only a few, although several excellent collections of these do exist (*1*, *2*). The physical properties of experimental diets (particle size, consistency, solubility, etc.), as well as such intangible characteristics as "attractiveness," taste, odor, and resemblance to natural diet, are frequently critical considerations. The diet must be adapted to individual feeding mechanisms. The role of symbionts is highly variable and must be assessed in each case. The chemical requirements of obligate symbionts are particularly difficult to assess. The nutritional requirements may vary during different stages of development, or for the germ as against the entire seed (as in plants), or for a tissue as against the entire organism. Finally, the criteria for nutritional adequacy are diverse and may involve growth, development, longevity, metabolism, reproduction, social behavior, or even such ill-defined concepts as "physical fitness" or "general well-being."

It is the purpose of this chapter to summarize the existing knowledge concerning the biochemical basis of the chemical requirements of living things. Such phylogenetic similarities and differences as are now apparent will be delineated, and the more significant voids in comparative nutrition will be emphasized. In keeping with this purpose, it will not generally be possible to discuss such other aspects of nutritional science as (*a*) deficiency symptoms, (*b*) factors affecting requirements, (*c*) nutritional interrelationships, (*d*) nutrient metabolism, (*e*) experimental techniques, (*f*) natural diets, and (*g*) feeding mechanisms, although key references which exist will be cited. Only brief reviews of comparative nutrition have previously occurred in the literature (*3, 4*), although more detailed reports for some specific groups have been published [bacteria (*5, 6*), fungi (*7, 8*), higher plants (*9, 10*), protozoa (*11*), crustacea (*12, 13*), insects (*14*), fish (*15*), mammals (*16–18*), including primates (*19*)].

B. Biochemical Function as the Basis of Nutritional Requirements

The chemical needs of living things are based upon various types of functional requirements. These functional requirements are sometimes fulfilled in different biological organisms by different kinds of chemical substances, that is, by different chemical species. It may be difficult, therefore, to compare, for example, the carbohydrate or the calcium requirements of diverse organisms, since these substances perform different major roles in different systems. It would thus appear expedient to compare chemical needs phylogenetically in terms of their specific biochemical roles. For example, the fulfillment of a given biochemical function may require certain specific chemical structural properties. The molecule possessing these properties may, however, perform other roles by means of its several functional chemical groups. Conversely, a number of different chemical species may have the proper structural characteristics to perform a given function. In some cases, indeed, abiogenic molecules which differ greatly from the naturally occurring ones may substitute in some functional sense quite satisfactorily for the natural nutrient [e.g., methylene blue for vitamin E (*20*)].

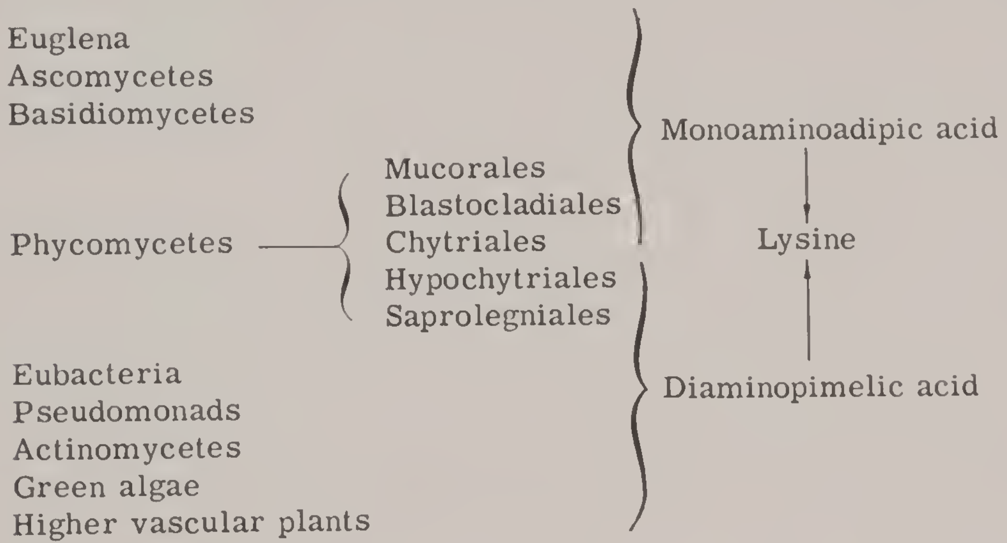
The biochemical basis of chemical needs may be considered functionally from the standpoint of the several different basic requirements for the life process, namely, maintenance of the environment, the source of free energy, structural organization, catalysis of chemical change in an essentially isothermal system, and certain more specialized biological activities. To this extent, at least, the chemical needs are reasonably clear cut, and it is in this sense that chemical requirements are conventionally considered in nutritional science. It should be pointed out, however, that other kinds of chemical needs for life processes do exist which are traditionally outside the scope of nutrition [e.g., the need of one tissue of a species for chemical substances supplied by other tissues of the same species (hormones, organizers, auxins); the need of one member of a species for chemical substances supplied by another member of the same species (sex attractants) (*21*); and conjugating and other chemotactic chemicals in many of the fungi (*22, 23*)].

C. NUTRITIONAL REQUIREMENTS AS A FUNCTION OF GENETIC CAPABILITIES

The great diversity in the chemical needs of the various forms of life is generally pictured as a consequence of the presence or absence of numerous biosynthetic enzyme *systems,* which, in turn, are a phenotypic expression of the genotypic composition of the species in question. Upon the primordial earth there existed only very simple substances, and it is presumed that these were converted stepwise by abiotic processes into the complex substances essential for the eobiont, a concept considered from the nutritional standpoint in Section II,B.

The chemical needs of autotrophic organisms which now exist are similarly met through the conversion of simple inorganic molecules by a stepwise, enzymatically mediated series of transformations. Thus, certain bacteria may oxidize iron or hydrogen to fulfill their energy requirements, and these substances along with the inorganic salts in their media constitute the only chemical needs, since from them the bacteria can synthesize all the more complex substances of which their cell is composed. When, through some genetic accident the organism is incapable of forming one enzyme in a sequence, precursors of a vital substance prior to that genetic "block" in the sequence are no longer nutritionally effective, whereas precursors subsequent to the ineffective site in the sequence will still meet the nutritional need. Thus with time and gradual genetic losses during evolution, nutritional requirements must become more complex. Biochemical evolution thus implies a loss in function in most cases and a greater dependence upon a specific nutritional milieu. Lederberg (*24*) has pointed out that "the main trend of biochemical evolution, from microbe to man, has been far less the innovation of new unit processes than the coordination of existing processes in space and time."

It occasionally happens that a single complex substance may be biosynthesized by more than a single sequence of metabolic reactions, so that organisms may sometimes survive a single genetic or chemically induced enzymatic block in one sequence. More often, but still infrequently, diverse pathways to a single product occur in different species. Since the genetic component of a species determines its identity, specific metabolic pathways (in addition to blocks that may occur in "single track" sequences) may be of aid in the identification of species and phylogenetic relationships through the study of the specific nutritionally active precursors of a single chemical requirement. This is well illustrated in the consideration of lysine biosynthesis by microorganisms (*25*), where the pathway for eubacteria, pseudomonads, actinomycetes, and higher vascular plants proceeds through monoaminoadipic acid, while that for the ascomycetous and basidiomycetous fungi and *Euglena* proceeds through diaminopimelic acid (I). Among the lower fungi (phycomycetes), there is a split; the Mucorales, Blastocladiales, and Chytriales proceed to lysine like the higher fungi, and the Saprolegniales and Hypochytriales behave more like the bacteria. Within the phycomycetes studied to date, the species taking the biosynthetic path common to the bacteria all produce biflagellate or anteriorly uniflagellate zoospores, while the species taking the pathway of the higher fungi all produce posteriorly uniflaggellate zoospores or aplanospores (Section V,A,12). It would appear that in more complex microorganisms where the total synthesis of lysine is not achieved, the nutritional needs of groups may differ, depending upon which pathway is involved, and that the two sets of enzymes "represent a considerable period of evolution and are viewed as biochemical organelles of ancient origin."



(I)

The gene-enzyme delineation of the organic metabolism of a species has evolved in the presence of various metabolically unrelated low molecular weight organic and inorganic substances, and, at some early time, metabolic activity became *dependent* upon such of these as were always present and structurally suitable to provide a more constant fluid environment, catalytic assistance, or morphological integrity to the organism (Section II,D). Thus the nutritional requirement for inorganic materials in any given species is similarly under genetic domination. Limited substitutions of these requirements may sometimes occur (e.g., bromide for chloride in activating salivary amylase), but these are usually inefficient on a quantitative basis.

Comparative nutrition shows promise of remarkable extension within the immediate future through the techniques of cell and tissue culture [mammalian (*26*), invertebrate (*27*), plant (*28*)]. Since cells under such conditions frequently undergo some loss in specialized function (e.g., reversion of osteoblasts to fibroblasts), it is sometimes difficult to decide whether to interpret the biochemical data in terms of morphologically similar but phylogenetically primitive species, or in terms of the parent of the culture. The latter alternative generally seems the most appropriate, however, in view of the genetic composition of the culture (even though it is not biochemically expressed). This concept appears to be supported by the observation that disaggregated embryonic tissues or organs, in the presence of a suitable nutritional environment, are capable of reassembling into organized groups resembling the original histological material (*29*).

D. Quantitative Evaluation of Chemical Needs

Whereas it is frequently easy to *assess* the quantitative nutritional requirements of a given species, it is generally exceedingly difficult and hazardous to attempt to *compare* such values for widely divergent phylogenetic materials, and the problem is usually proportional to the degree of divergence. Quantitation is in terms of amounts per day per unit weight for many higher species, in fractions of the total food intake for insects, farm animals, fish, and some other animals, and in concentration in the fluid medium for most aquatic organisms. None of these have a very direct relationship to the function of the nutrient in the life process. Varying types of assimilative, digestive, absorptive, and distributive mechanisms further aggravate the problem of comparisons. Tissue culture studies, of course, despite some limitations on their over-all biological informativeness, obviate many of these problems.

In making quantitative comparisons between animals, it is obviously of extreme importance to consider the probable major *qualitative* function of a given nutrient, and insure that this relationship holds in the species under study. It would be senseless, for instance, to compare the calcium requirements of the mollusks without first segregating the species on the basis of whether or not they possess a calcareous shell. In less obvious instances and in the absence of prior knowledge, however, such a comparison would of course bring out through anomalous values the occurrence of special functions for a nutrient in a given species in the series studied.

When the purpose of quantitating comparative nutrition is primarily ecological, the diverse units of measurement previously mentioned may be adequate, but they are clearly inadequate in considering chemical requirements in terms of biological function. In the latter instance, it is more appropriate to consider the amount of the nutrient *ingested* (or even better, *absorbed*) in terms of its specific function. For vitamins A or D for instance, quantitation might appropriately be in terms of some function of photoreceptive activity, or calcium transport or relative skeletal mass, respectively. Thus, despite much evidence to the contrary, it was suggested on a quantitative nutritional basis that tocopherol did not belong to this special-function, fat-soluble group of nutrients, but rather was involved in intermediary metabolism like the B vitamins (*30*); this preceded by many years the final confirmation of this fact.

Various methods have been devised for relating nutrient requirements to metabolism and growth when the nutrient is primarily involved in these processes. Most commonly such chemical needs vary with some function of respiratory exchange, expressed upon a weight or unit surface (weight ⅔) basis per unit time. It has been shown (*31*) that, over the broad spectrum of the animal kingdom, the respiration (and thus the requirement) varies among different species with either the surface alone (mammals, crustaceans, clams), the weight alone (growing insect larvae, annelids), or directly with one *and* at the same time reciprocally with the other (certain pond snails and flatworms). Nutritional requirements may also be related to the growth rate, although in the human species the growth curve is distorted from that followed by most other species by the long period of youth (*31*).

E. The Nutritional Economy of the Biosphere

The chemicals which meet the nutritional needs of the total terrestrial biota are distributed within a thin shell above and below the surface of the earth. The total amount of carbon in this biosphere is estimated to be about 2.8×10^{17} gm., so that the total mass of the biosphere is about ten times this, or 3×10^{18} gm. (*32*). Photosynthesis upon the earth produces about 2.5×10^{17} gm. of glucose annually, consuming about 3.6×10^{17} gm. CO_2 in the process (*33*). Changes of like magnitude occur in the chemistry of many of the other elements which compose living material (e.g., oxygen, nitrogen, sulfur, and phosphorus), and these are primarily mediated through biological processes. Nonbiological physical and chemical forces at the present time play a relatively insignificant role in these events. It is apparent that photosynthesis alone would soon exhaust the available supplies of CO_2 in the biosphere, and that nonphotosynthetic life, if similarly left alone, would soon exhaust the available supplies of molecular oxygen and reduced carbon compounds. Any sudden change in this delicately balanced, solar-powered cyclic series of changes, even if slight and momentarily imperceptible, would rapidly become catastrophic. Not the least of the delicate steps in this balance is the ability of plants to accumulate and concentrate carbon from its very low atmospheric concentrations. Some slight amount of carbon continually enters the atmosphere through the weathering of rock and by extraterrestrial influx, but large amounts leave the biosphere and are more or less lost to it in the form of petroleum and other carboniferous deposits (*34*), which may be thought of as terrestrial storage forms of solar energy.

The nitrogen balance of the biosphere is similarly maintained by cyclic biological events involving oxidation of NH_3 to N_2, NO_2, and NO_3; reduction by the opposite sequence; conversion to organic forms; and deaminative changes to the inorganic. Without nitrogen-producing bacteria, nitrogen-fixing organisms would at their present rate remove all nitrogen from the atmosphere after a few million years. If only chitin formation continued at its present rate, and nitrogen-fixing bacteria were adequate to continue the task of providing nitrogen compounds for its synthesis by chitinous animals, atmospheric nitrogen would all pass into chitinous nitrogen in about 41 million years (a short time in the total span of life on the earth), were it not for the intervention of chitin-splitting bacteria (*35*). The maintenance of appropriate nutritional supplies of nitrogen compounds is thus a delicate, complex biological phenomenon.

An involved series of changes inextricably links the distribution of phosphorus on this planet with its critical nutritional requirement for energy transfer and storage in all known terrestrial forms of life. Biotic activities profoundly influence its precipitation and solution and may cause its concentration in guano deposits or in mineral deposits consisting of the calcareous remains of a multitude of plant and animal species (*36*).

The nutritional supply of terrestrial sulfur would similarly be handicapped were it not for its biological oxidation from sulfides to elemental sulfur and sulfates, its reduction from sulfates back to sulfides, and the transformation of these forms to and from organic derivatives. These biological processes result in such tremendous geochemical events as the massive erosion of rock through H_2SO_4 formation, and the deposition of huge deposits of elemental sulfur and metallic sulfides. The nutritional availability of assimilable forms is nevertheless maintained, apparently through runoff of terrestrial sulfates into the sea, utilization of the soluble sulfate by plankton, and, through biological reduction, the formation of H_2S which rises into the atmosphere and is transported back to the land where it is oxidized by the "sulfur" bacteria to complete the cyclic series of events (*37*).

The evolution of the nutritional requirement for minerals is similarly linked with biogeochemistry, and pertinent studies have been made on calcium, iron, copper, manganese, vanadium, and others (*34*). Adoption of these and related mineral elements as functional and nutritional requisites in primitive biota, and rejection of other elements, appears to derive from: (*a*) their concentration and distribution in the lithosphere; (*b*) their equilibrium concentrations between the lithosphere and hydrosphere; and (*c*) their chemical suitability for a specific function (e.g., possession of low atomic weight, polyvalency, electrochemical properties, ability to form coordination complexes, capacity to form soluble or insoluble combinations, ionizability, amphoterism, compatibility with other ingredients of the milieu, etc.). The evolution of mineral nutritional requirements was an inevitable consequence of the terrestrial environment at the biopoietic moment, and essentially identical mineral requirements would evolve in neobiopoiesis on this or other planets when environmental conditions were similar. The evidence as to whether or not this is so with regard to the complex *organic* requirements for biopoiesis must, therefore, next be reviewed.

II. The Abiogenic Fulfillment of Biopoietic Demands

A. Origins of Terrestrial Life

Theories of the spontaneous generation of complex forms of life died with the fermentation experiments of Pasteur, which demonstrated that the techniques used by workers attempting to prove spontaneous generation were inadequate for that purpose. Neobiogenesis was *not* disproved by these studies, however, nor has it been subsequently. The concept of the origin of exceedingly simple primitive forms of life from inanimate material (biopoiesis) arose late in the nineteenth century but received little serious attention prior to the publication of Oparin's "Origin of Life" in 1936 (*38*). Subsequently, a large and now rapidly increasing volume of experimental work has been published (*39*) which suggests that the synthesis of living material from substances formed by abiotic processes is not as improbable as once appeared. The present consensus is that life first arose *de novo* upon the earth at some distant time (ca. 2.5×10^9 years ago), and that all subsequent life has descended from the product of this event. Some possibility exists, however, that the process has been repeated many times on the earth and presumably other planets even unto the present. As against this is the less widely held panspermia hypothesis of Arrhenius (*40*), which proposes that life was transferred to this planet from outer space, possibly in the form of spores. This latter proposal, however, still evades the basic problem of the nature of life's eventual origin. Relating to this, the startling thought has been advanced that the first life was transported to this planet by intelligent beings (*41*). The concept of a *spiritus vitae* in living things has largely disappeared from current thinking.

Major interest in the biopoietic problem stems from the question of how the complex organic materials of which the eobiont was composed could arise in the absence of life, since these substances do not *appear* to arise in nature by abiotic processes at the present time. Many practical answers to this question have already been provided. The formation of these original "nutrients" and their conversion to more complex substances or their high molecular weight polymers in the absence of any form of life appears to have followed the same pathways that we now recognize in intermediary metabolism. The subject is of interest in the present discussion because it represents, in fact, the origin of the nutritional process itself.

The time sequence in biopoiesis remains uncertain, but it is generally believed that biopoiesis occurred in the following stages: (*a*) formation of the most primitive organic compounds (e.g., hydrocarbons); (*b*) their transformation to relatively simple molecules, such as amino acids or trioses; (*c*) subsequent polymerization to proteins, polysaccharides, or nucleoproteins; (*d*) aggregation of these materials into the eobiont; and (*e*) the evolution of metabolism.

B. The Primitive Biosphere

When the earth was formed about 5×10^9 years ago, its chemical composition probably approximated that of the sun, but during its early history much of its original mass was lost. The minute quantities in our atmosphere of the heavy gases xenon and krypton (as compared with concentrations in the sun and the rest of the universe) suggest that these substances were distilled off into space by diffusion of the lightest element, hydrogen, which accounts for 2.7 atomic per cent of the earth's crust, as against 81.76 atomic per cent of the sun's protosphere. This loss of hydrogen accounts for the decrease in mass of the earth and was due to the temperature and mass of our protoplanet. (In general the concentration of hydrogen in the atmosphere of our planets varies directly with the planetary mass.) By contrast, the atomic percentages of oxygen for the earth and the sun are 48.7 and 0.03, respectively, so that the earth's atmosphere must have changed from a reducing atmosphere to an oxidizing one. Since the original material of the earth's atmosphere was lost, the oxygen of the air and seas must have arisen from the water vapor released from the earth's interior during its cooling (free oxygen cannot escape from the interior). The portion of this water below the tropopause underwent photolysis, and the escape of hydrogen from the atmosphere at this period brought about a consequent enrichment with oxygen, later to be increased to present levels by photosynthesis. In a similar manner, methane and ammonia were decomposed, by solar radiation, and their decomposition products dissolved rapidly in the surface waters. Aboriginal carbon dioxide reacted with silicate rocks forming limestone, sulfides were oxidized to sulfates, and metals to their oxides, all at the time of the event of the oxidizing atmosphere. But the molecular oxygen thus formed absorbs in the photolytic region (<1850 Å.) of solar radiation, so that the rate of the dissociation of water vapor and subsequent hydrogen loss from this planet greatly decreased. Thus arose the perhaps unique oxygen content of our atmosphere.

Volcanic gases of today give some indication of the composition of the early atmosphere, and consist largely of water vapor, with lesser amounts of CO_2, CO, HCl, HF, H_2S, N_2, NH_4Cl, and possibly CH_4 (CH_4 arising by reduction of C, CO, or CO_2 at the high temperatures present). The interaction of these latter compounds and their breakdown products produced a variety of substances in the primitive oceans, but aside from these there was little other dissolved material. The anions of the surface waters arose as part of the escaping gases from the internal rocks of the earth's mantle (carbonate, fluoride, chloride, sulfate, borate, phosphate, etc.); by contrast, the cations arose by weathering of the surface rocks (sodium, potassium, calcium, magnesium, etc.). The heat of the earth approached present levels ca. 2×10^9 years ago; the last major changes in composition occurred in the atmosphere and oceans ca. 2 to 3×10^9 years ago; the first forms of life appeared ca. 1.5 to 2×10^9 years ago. The coincident appearance of water, molecular oxygen, and a moderate temperature were the major factors that determined the creation of the biosphere, and subsequent biogenesis.

C. Formation of Simple Organic Molecules

A great deal of evidence has been presented to show that the simplest of organic compounds which must undoubtedly have occurred in the prebiotic world can, when activated by an energy source, be converted to more complex ones. Miller (*42*) obtained a variety of amino acids and other organic compounds by the use of an electric discharge in methane-ammonia-hydrogen mixtures (Table I). Ammonia and formaldehyde mixtures under the influence of ultraviolet radiation have given successful yields of amino acids in like manner, as has the use of heat and of various inorganic substances acting as catalysts. Further, for instance, malic acid and urea react to give aspartic acid which in turn reacts with urea to give ureidosuccinic acid, a recognized pyrimidine precursor (*43*). In brief, it may be said that all the substances that are

TABLE I

Products Of The Action Of Electric Discharge
In A $CH_4 : NH_3 : H_2$ Mixture[a] (Miller)

Compound	Yields of compounds (moles x 10^5)	Compound	Yields of compounds (moles x 10^5)
Glycine	63 (2.1)[b]	Formic acid	233
Alanine	34	Acetic acid	15.2
Sarcosine	5	Propionic acid	12.6
β-Alanine	15	Glycolic acid	56
α-Aminobutyric acid	5	Lactic acid	31
N-Methylalanine	1	α-Hydroxybutyric acid	5
Aspartic acid	0.4	Succinic acid	3.8
Glutamic acid	0.6	Urea	2
Iminodiacetic acid	5.5	Methylurea	1.5
Iminoaceticpropionic acid	1.5	Sum of yields of compounds listed	15 %

[a] From Miller (42).
[b] Percentage yield of glycine, based on carbon placed in the apparatus as methane.

necessary for production of amino acids, simple saccharides, fatty acids, and purines and pyrimidines by abiotic methods have been prepared by these techniques and that in many cases these biochemicals themselves have been prepared directly in this fashion. There consequently appears to be no fundamental reason to doubt that low concentrations of the fundamental "building blocks" of protoplasm did appear in the prebiotic biosphere and that they are still being formed in this manner. Although primary study in this area has been directly toward amino acid production, it seems likely that the formation of compounds of the keto acid-alcohol type preceded these upon the earth, particularly since these latter substances readily interact at room temperature to produce an apparently limitless variety of derivatives to form the protein progenitors.

One of the basic problems encountered in the study of the abiotic synthesis of organic compounds is that of asymmetry, which normally does not appear in laboratory syntheses and which is a common property of the products of biological synthesis. Among the factors that may have been involved in primordial asymmetric syntheses are (*a*) resolution of racemic mixtures by spontaneous crystallization of one antipode (e.g., DL-glutamic acid can simply be resolved by seeding with one enantiomorph), (*b*) the chemical action of circularly polarized light, and (*c*) asymmetric catalysis with dissymmetrical mineral crystals (39). It is generally supposed that the synthesis of amino acids must precede polymer formation, but this is not necessarily true. It can readily be shown, for instance, that the polymerization of aminoacetonitrile (produced by the condensation of CH_2O, NH_3, and HCN) followed by hydrolysis will result in a polyglycine chain. By condensation with aldehydes or unsaturated hydrocarbons, this would yield a protoprotein, which upon subsequent hydrolysis would produce amino acids with a common steric configuration, if the polyglycine had been adsorbed onto a clay surface during modification (*44*). The possibility further exists that both enantiomorphs of a compound may have occurred in primitive organisms and that one form won out by a type of natural selection on a molecular level (*45*), but this theory is not generally considered to be tenable.*

D. Formation of Complex Organic Molecules

The simpler organic molecules may readily react by decarboxylation, deamination, oxidation, reduction, and condensation to form an infinite variety of derivatives at room temperature in the presence of a large number of naturally occurring inorganic catalysts. It is noteworthy that the condensations to form peptides, glycerides, polysaccharides, and polynucleotides are all dehydrations that readily occur at elevated temperatures in the absence of other factors. It is also important to recognize that the order in which different monomers appear in a polymer need not be controlled by extraneous factors, since structural considerations alone will control this somewhat. Furthermore, the presence of a single ordered polymeric strand acting as a template greatly facilitates the production of a second one in the same order, as shown by the pairing of adenine-thymine and guanine-cytosine in nucleic acid replication. Since complex molecules are more resistant to heat than simpler ones, syntheses are biased toward the net production of the most complex ones.

It is generally proposed that the complex reactions involved in the production of polymers, as well as the condensation of various such polymers to form the probiont, was facilitated by the build up of concentrations of basic organic molecules in the primitive oceans to a high concentration ("soup") over a long period of time. The rapid exhaustion of this organic sea by the explosion of life might then obviate the possibility of recurrent biopoiesis. It has been pointed out, however, that concentration is not dependent upon the "soup" hypothesis, since it can readily be achieved from very dilute solutions by adsorption onto the various surface-active clays and other minerals present, and that the material can be polymerized in that adsorbed state (*46*).

Many of the properties of complex polymeric systems are undoubtedly the characteristics of subtle variations in primary and secondary chemical bonding. Thus heating at 70° of mixtures of eighteen amino acids in the dry state and in the presence of phosphates yields proteinoids containing all of the amino acids and possessing virtually all of the properties of natural proteins, including a degree of orderliness in arrangement (*43*). These substances, when placed in solution, have a tendency to yield microspheres resembling cocci, with diameters in the bacterial range; the spherules shrink in NaCl solutions hypertonic to that in which they were produced (osmotic boundaries). They vary in size and shape depending upon their composition and the presence of other substances (e.g., lipids). In regard to the behavior of these preparations, Fox (*43*) has pointed out:

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* Editor's Note: For other views on this subject, see Chapter 1, by Ulbricht, in Vol. IV of this treatise.

> The first sudden production of osmotic boundaries created not only the form of the most luxuriant form of life that we know, but the first environment. Some of the first prebiological units could do prebiological business, and subsequently biochemical business, with that environment. The universal necessity of nutrition for all cells has been with us ever since. . . .

Allusion should be made at this time to the problem of the origin of viruses. In this instance we have an apparently modern analog of the eobiont, with nutritional requirements for all the basic components of which they are composed. Their similarities are suggested by Keosian (*47*) in his emphasis on neobiogenesis:

> The difficulty of placing viruses, bacteria, certain "algae," sponges, and so on in a fitting place in any taxonomic scheme based upon a monophyletic hypothesis may stem from a possibility that the discontinuities are real and represent the existence of separate lines of descent from independent instances of neobiogenesis at different times in the history of the earth down to the present.
>
> Concretely, it would appear more plausible to accept present-day viruses as units of recent and present day origin than to suppose that they descended through some two billion or more years relatively unchanged. Throughout time, viruses either evolved into higher organisms or were eliminated in the process of evolution, being ever re-established through neobiogenesis.

E. Nutrition on Extraterrestrial Bodies

Within our galaxy alone there are about 10^{11} stars, and it is believed that the majority of these have a number of planets. If each star had just one planet within its ecosphere (the life-permitting zone), the probability is very high that life exists on many other bodies in our galaxy. The improbability of the uniqueness of terrestrial life is further confirmed by the facts that: (*a*) our sun has four planets within its ecosphere; (*b*) there may be habitable moons; (*c*) subsurface life may extend the ranges of the ecosphere; (*d*) life in outer space may evolve on other more thermostable chemical principles; and (*e*) there are billions of galaxies other than our milky way. Interest in this extraterrestrial life is important for a number of *nutritional* reasons. The forms of life on other planets will enable us to see whether terrestrial nutrition is unique, or representative of a universal principle. It may also make possible the direct study of the evolution of our own nutritional patterns through the examination of primitive forms no longer present on the earth. Finally and from an immediate and practical standpoint, it is desirable that we investigate the possibility of fulfillment of our own needs [the fitness of the environment (*48*)] on extraterrestrial bodies, either directly or through the establishment thereon of a nutritionally adequate and biologically specific immigrant biota.

The planet Mercury is not generally considered to be within the ecosphere, although its dark side, which is reasonably cool, may have accumulated considerable amounts of organic matter and have an indigenous or panspermic biota, or at least be habitable (*49*).

Venus probably has a prohibitive surface temperature of around 6000° K., and so contains no surface water, but its atmosphere contains an abundance of N_2 and CO_2, and probably an ice-crystal cloud layer at about 30–40 km. above the surface. The oxidation level is undoubtedly much lower than that of the earth, and there is no good evidence that life now exists on this planet. The prospect of microbiological planetary engineering has, however, been considered (*50*), and it is suggested that blue-green algae of the Nostocaceae may be nutritionally and metabolically adequate to the task. Thus the properly selected, temperature-resistant, aerial species may survive at the lower temperatures of the higher levels, utilizing the cloud-layer water crystals, fixing atmospheric nitrogen, fixing CO_2 through photosynthesis, and evolving O_2. Mineral requirements might be supplied through the dust of meteoric infall. In time the organic material formed would fall to the surface where it would be burned to liberate carbon and water. Oxygen would accumulate, CO_2 would decrease, the surface temperature would fall sufficiently to permit of liquid water, and surface photosynthesis would initiate the formation of a reasonably habitable environment for a more varied biotic collection.

The atmosphere of Mars also contains predominantly nitrogen, considerable amounts of CO_2, and small amounts of water; the surface temperature ranges from about −70° to 30° C. The presence of molecular oxygen is uncertain. The most plausible explanation of certain astronomical data is that Mars is now a life-bearing planet; it is probable that many simple organisms present on the earth could survive there, and many workers feel that Mars does have its own biota, either indigenous or panspermic in origin (*49*).

The problem associated with biological contamination of the Moon by terrestrial missels, or by natural projectiles from interstellar space, has attracted much attention to the conditions that exist on its surface (*51*). For some time after its formation it probably possessed a secondary atmosphere of CH_4, NH_3, H_2O, H_2, and their interaction products. Complex organic substances were probably formed from these materials, in the manner previously described for the prebiotic earth, so that below the subsequently deposited meteoric dust layer it has been estimated that there now exists a complex of organic matter having a mean surface density of approximately 10 gm. $cm.^{-2}$ (*52*). Surface temperatures range from 100° to —180° during a lunar day and night, but a few centimeters below the surface the temperature is much more moderate. It has been estimated that the odds against contamination of the Moon by extrasolar system astroplankton are tremendous (*53*), but that the odds of contamination from planets within our own solar system are great and that extinct terrestrial microorganisms may still be found there.

III. Requirements for Maintenance of the Fluid Medium

A. General Consideration

The primitive seas provided an immediate and direct source of nutrition for their simple biota, but as organisms became more complex, some of their cells could not have survived without the evolution of open channels through which the water could penetrate to cells within the center of the tissue masses. Subsequent removal of these channels from direct contact with the external environment provided the first closed circulatory system and allowed greater independence from the vicissitudes of environmental changes (*54*). It also created additional problems in obtaining nutrients from the external environment, since these substances were required to pass from the external environment through an intermediate barrier to the metabolic area of the organism. Even in the most complex organisms, the osmotic pressure of this intermediate barrier, mediated by the electrolytes of the original marine waters, remains a considerable factor in maintaining the integrity of the intracellular fluids. Selective absorption developed, however, to modify the fundamental osmotic mechanism, and a variety of physiological devices evolved to meet individual needs and to provide even greater independence from the environment. From a biochemical standpoint, the adoption of osmotically active organic solutes was a major advance in some groups of plants and animals, since it heralded a phase of almost complete independence from the obligately high nutritional requirement for the original marine electrolytes. At the present stage of evolution, in most organisms, nevertheless, the greater portion of the quantitative requirement for minerals is still attributable to the need for regulation of the internal environment. The mechanisms for osmoregulation, as distinct from the chemical requirements for this process, have become varied and complex; they are discussed in Chapter 9 of Volume II of this treatise.

B. The Plant Kingdom

The pH of sea waters most often ranges from 8.0 to 8.4, but the pH of most land plants is slightly acid. The range of pH in plant saps may vary considerably, being as low as 0.96–1.36 for *Begonia* "Lucerna" and 1.70 for *Oxalis* sp., but more generally ranging from 5.0 to 6.5 (55). The lower plants have an even greater pH tolerance, *Thiobacillus thiooxidans,* an extreme case, growing best in media having a pH of 2.0–3.5 (*34*) and surviving at pH values of 0.2–0.6. The pH optimum for the fungi is lower than that for most of the true bacteria, but seldom falls below 3. The slightly acid pH of most plants appears to be a reflection of the large amounts of organic acids that they may accumulate and would be reflected nutritionally by only slightly increased needs for cations. The low pH of *Begonia* sap, most likely due to differential ion exchange, must require considerable amounts of buffer (inorganic or organic) for maintenance of intracellular processes; the low pH of the thiobacilli media is due to the oxidation of sulfur to sulfuric acid, and again intracellular buffer requirements must be high. In this regard, it is of some interest that plant fluids are least strongly buffered at their natural pH, their buffering capacity rising toward both the extremes of the pH range.

The osmotically active constituents of higher-plant cell sap consist largely of glucose, or organic acids derived from it, and of potassium salts of the latter and of phosphate. Glucose and its derivatives are of course products of photosynthesis, but their requirement in the nutrition of etiolated plants is difficult of quantitative evaluation. As early as 1865 Knop demonstrated that normal plant growth could be sustained indefinitely upon the following minimal medium:

$Ca(NO_3)_2 \cdot 4H_2O$	0.8 gm./liter
KNO_3	0.2 gm./liter
KH_2PO_4	0.2 gm./liter
$MgSO_4 \cdot 7H_2O$	0.2 gm./liter
$FePO_4$	trace

Trace elements were, of course, inadvertently present, but the nonessentiality of sodium and chloride was demonstrated. The latter ions are present in considerable amounts in some aquatic plants and are thus osmotically active, even if unessential; sodium is essential, however, for the blue-green algae (*56*). Calcium and magnesium, though essential for higher plants, may play only a minor osmotic role. Calcium plays only a trace element role in some fungi and is apparently nonessential in most bacteria. The role of inorganic sulfate in plants (and animals), though not manifest in high tissue levels, may be significant because of its convertibility to organic forms.

Maintenance of the intracellular environment of lower plants is poorly understood, perhaps owing to their relatively high resistance to osmotic changes, but it seems probable that organic solutes play a major role in this phenomenon, thus providing greater independence from the immediacy of environmental changes which confront them. Some electrolyte component does, however, remain essential, and data may be cited for many species. *Aspergillus niger*, for instance, requires about 1.5 gm. of potassium and 0.5 gm. of magnesium to produce 100 gm. of mycelium and requires an optimal level of 150 mg. of potassium per liter of medium (*57*). Sodium and beryllium may supplement suboptimal levels of these elements, respectively, but will not entirely replace them. Sodium, potassium, magnesium, iron, chloride, sulfate, and phosphate are required by *Escherichia coli* (*58*), but there is no evidence as to their exact functions.

Many marine bacteria may be regarded as salt-adapted forms of freshwater types. Indeed the freshwater *Desulfovibrio desulfuricans* can be gradually adapted to increasing salt concentration (*59*), becoming what was once thought to be a separate species, *"Vibrio aestuarii."* Studies of obligately halophilic bacteria are of some added interest in this regard, since these require media containing over 2.5% salt and are cultured in media containing 10–15% salt (*60*). These too may be regarded as adapted forms. For some (e.g., *Vibrio costicolus*), regular and measurable growth responses occur with increasing amounts of sodium chloride, but other salts may substitute for this completely. With others (e.g., *Micrococcus halodenitrificans*) this substitution is not possible. In general, however, it may be concluded that, for the halophiles at least, sodium and chloride ions both have a beneficial effect, that of sodium being the more important. Sugar solutions will not supplant the salt requirement for *V. costicolus*, however, nor are sugar solutions isotonic with sea water satisfactory for maintaining the osmotic relationships of marine organisms. It thus appears that there exists both a certain particle concentration required to obtain optimum osmotic pressure, and a specific electrolyte requirement concerned with other biological processes.

Associated with microbial facultative or obligate halophilia, and related, but in a completely unknown manner, is the phenomenon of pigment formation (*61*). *Bacillus cereus* var. *mycoides*, a normally colorless soil species, produces red and yellow carotenoid pigments when grown in media containing 32% NaCl; these are similar to those found in *Halobacterium halobium* isolated from salted fish. *Pseudomonas beijerinckii* forms a hydroquinone pigment, but only when grown in 12–18% NaCl. About two-thirds of the marine micrococci are yellow or orange forms, and the most prominent algae of salt-evaporating pools (NaCl saturated) are bright red (*Dunaliella salina*).

C. THE ANIMAL KINGDOM

The pH of the internal media of animals is generally near the neutral point (*62*); land vertebrates average about 7.4, marine animals from 7.2 to 7.8, and insects slightly below 7.0, making them unique. These values are maintained by buffering substances which, with the major exception in some instances of the proteins, are practically identical with the materials that maintain osmotic relationships. The *milieu intérieur* of higher animals is best buffered near the natural pH, while that of insects, like plants, is better at the extremes (*63*).

Osmotic and electrolyte balance in the animal kingdom is generally achieved through the action of sodium, potassium, calcium, and magnesium as cations. Chloride, bicarbonate, sulfate (*64*), phosphate, organic acids, and protein act as anions. Exceptions have been discovered in the case of the elasmobranchs, where trimethylamine and urea play an important role, and in insects, where amino acids and other organic solutes achieve high levels in the hemolymph. It is a general truth that among animals sodium is the chief extracellular cation, and, along with calcium, it is balanced against potassium and magnesium as the main intracellular cations. Chloride and bicarbonates predominate as anions outside of the cells (except in insects), while intracellular anions are predominantly phosphates, sulfates, and proteins. Ratios of cations to anions are important in maintaining pH levels, and ratios of monovalent to divalent cations are of special significance with regard to tissue "irritability" in animals, a problem that is not as marked in the plant kingdom. The ratio of $(Na + K):(Ca + Mg)$ is much lower in the fluids of invertebrates than it is in the vertebrates, regardless of the habitat or mode of life. This ratio for man is about 21.7 and for other vertebrates seldom as low as 12.0; it does not exceed 6 or 7 in invertebrates (*65*). The lower relative amount of the alkaline earths in the vertebrates may be associated with the large amounts of calcium and magnesium stored in the endoskeleton of this group. It is of some interest that the electrocardiogram of a snail heart placed in fluid containing these cations in ratios similar to those of vertebrates resembles the electrocardiogram of a vertebrate, whereas the electrocardiogram of a frog heart placed in a medium suitable for invertebrates becomes more primitive (*66*).

The total osmotic pressure of the blood of marine invertebrates approximates that of sea water, while that of freshwater invertebrates is much lower, but above that of fresh waters. Somewhat above that of the latter group is the osmotic pressure of marine *and* freshwater teleost fishes, amphibians, birds, and mammals. The *inorganic* component of the osmotic pressure of elasmobranchs is about the same as for mammals, etc., but the presence of additional organic solutes brings the total value still higher for freshwater elasmobranchs and to values even exceeding that of sea water for marine species. Within these ranges, values may vary within a species depending upon developmental stages, as with the larval and adult stages of insects; and between species depending upon habitat (e.g., osmotic values of many parasitic organisms approach those of the host).

The nutritional requirements for the maintenance of the internal environment of animals may thus be summarized as consisting of sodium, potassium, calcium, magnesium, chloride, phosphate, and possibly amino acids. From a quantitative standpoint, the requirements for these ions among the different species vary with the factors previously mentioned and with the numerous regulatory mechanisms provided in the animal kingdom for stimulating or depressing the absorption or excretion of each ion. Regulation of water metabolism may also be an important factor in maintaining osmotic relationships, and under conditions of acute water shortage metabolic water may supplement the nutritionally limited supplies. In animals so handicapped, there is a strong tendency to metabolize fat, which produces over twice as much water (107 gm. per 100 gm. of fat) as do carbohydrates or proteins, but in this case with no demonstrable ketosis (*67*). Specific values for inorganic nutritional requirements may be found for some of the more common species in the general reviews of their nutritional requirements (*5–19*).

Special mention should be made of the organic nutritional requirements for osmoregulation. The urea and trimethylamine levels of elasmobranch plasma are readily supplied in the processes of intermediary metabolism, and are thus not manifest in the chemical needs of these fish. The bicarbonate component of the plasma of all animals is also supplied metabolically, so it is not a dietary requisite. In this latter case it is of some interest that the carbon dioxide content of tunicate blood is even less than that of the water in which the animal lives (*65*), a reflection of the small alkaline reserve of these species which characterizes them chemically.

In the insects more than any other group studied, there appears the greatest diversity of osmotic solutes (*68*). This may, however, be a reflection of the more intensive study of this group. Blood uric acid levels are higher in insects than in other species (insects 10.5–14.5 mg.%; crustacea 0.3–0.9 mg.%; traces or none at all in other invertebrates; 2–6 mg.% in humans). The level of reducing, nonfermenting substances is also high in insect hemolymph, but this is probably attributable to a mixture of such substances as uric acid, ascorbic acid, amino sugars, tyrosine, and other phenolic compounds. High amino sugar levels have been reported in some species, a possible reflection of the chitinous nature of these animals. Insect blood is characterized also by being particularly rich in magnesium and phosphate, as contrasted to other animals, and frequently potassium levels also are high. Zoophagous insects have high sodium levels, and phytophagous insects high potassium levels. In this regard, dietary considerations may be related to phylogeny (*69*). The primitive pattern is believed to be a high sodium type requirement (and high hemolymph concentrations), as seen in the Odonata, and in modified form (e.g., with high magnesium) in many Orthoptera and Diptera and some Coleoptera. The wingless thysanurans apparently follow this pattern with a high Na:K ratio. In more advanced forms, evolving along with the angiosperm plants, there is adaptation to the plant diet with increased potassium and magnesium levels. In insects, chloride is seldom the predominant anion as it is in vertebrates (*69*), being supplanted to some appreciable extent by organic acids and phosphates.

Amino acids in insect hemolymph act predominantly as cations, basic amino acid(s) being abundant, as compared with aspartic and glutamic acid. Whereas free amino acids occur in most animal plasmas in ranges of from 50 to 100 mg.%, in insects the range is from about 300 to 2400 mg.% (*70*). More primitive orders of insects tend to have the lower levels within this range. Most species have high concentrations of glutamine (but not the corresponding acid) and proline, and one or more of the basic amino acids. Relative hemolymph levels of the different amino acids provide a quite constant and characteristic pattern for a given stage of development of any species; species differences are great, however, so that these patterns may have taxonomic value. Feeding of various pure amino acids has been shown to alter the blood levels, so that in many instances the amino acid levels in hemolymph must have a definite effect on the nutritional requirement for these substances. The requirement is further increased when the levels in the hemolymph are drawn upon for special syntheses, as in the silkworm.

Numerous carbohydrate derivatives occur in the hemolymph of some insects in quantities sufficient to be osmotically active (*68*). The principal blood sugar in insects is trehalose, levels varying greatly but commonly ranging from 1 to 6%. Precise amounts are quite variable and dependent upon nutritional status, since ingested glucose, fructose, or mannose is rapidly converted to trehalose. In adult bee hemolymph, reducing sugars (glucose, fructose) occur at levels of up to 4%, but somewhat lower levels of monosaccharides occur in most insects. Citrates occur generally, reaching a level of 96 meq. per liter in silkworm larval hemolymph. Malate, succinate, and lactate have also been reported in this species. In *Gasterophilus* larvae, these acids along with fumarate and ketoglutarate account for 50% of the anion level. Levels of glycerol may reach very high levels at certain stages in the development of some insects (25–30% of whole insects), and are believed to be associated with cold-hardiness (*71*).

IV. The Nutritional Source of Free Energy

The *immediate* source of free energy for vital processes in all known forms of life resides in the covalent bonds of monosaccharides. Within the animal kingdom, and among the heterotrophic plants (i.e., many bacteria and fungi), there is a nutritional requirement for these monosaccharides or for other reduced forms of carbon which are directly convertible to them. Among the nonphotosynthetic autotrophic bacteria, the oxidation of inorganic substances provides the source of free energy for the reduction of carbon dioxide to form the requisite monosaccharides. In the photosynthetic algae, bacteria, and higher plants, the capture of solar radiation makes available the necessary energy for the reduction of carbon dioxide to form the saccharides which are required for immediate energy in plant metabolism (and as a nutritional source of energy for heterotrophic organisms). On the basis of the nutritional source of carbon and of free energy, therefore, living organisms may be classified as (*a*) photosynthetic autotrophs, (*b*) chemosynthetic autotrophs, or (*c*) heterotrophs.

A. PHOTOSYNTHETIC AUTOTROPHS

The mechanisms of photosynthesis are discussed elsewhere in this treatise,* and the nutritional requirements for the process are presented below in this chapter. It will be recalled that during the early history of the earth, the energy of solar radiation in the range of 1850 Å. was captured by the direct photolysis of water vapor and perhaps of other simple substances but that this process was terminated by the accumulating molecular oxygen content of the atmosphere, which absorbs in this same wavelength range (cf. Section II,B). Biological photosynthesis is an evolutionary adaptation of this process, in which solar radiation of longer wavelength is captured by carotenoids and chlorophylls and the energy is utilized in the dissociation of water. The atomic hydrogen which is formed, or its equivalent in protons and electrons, is transferred to an as yet unidentified primary hydrogen acceptor (chlorophyll, dehydrolipoic acid?), and eventually forms DPNH. The DPNH is utilized in the reduction of 1,3-diphosphoglyceric acid, forming 3-phosphoglyceraldehyde in the dark reaction. The primary CO_2 acceptor in the dark reaction is ribulose-1,5-diphosphate, and the CO_2 addition yields two molecules of phosphoglyceric acid, which must be reduced by DPNH to the aldehyde. Subsequent steps in the formation of hexose must also regenerate the original ribulose-1,5-diphosphate, so that the sequence is much more complex than the simple reversal of glycolysis. The net reaction may be written,

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* See Chapter 5, by Wassink, in Volume V of this treatise.

$$\begin{aligned}&6\ \text{Ribulose-1,5-diphosphate} + 6\ CO_2 + 18\ \text{ATP} + 12\ \text{DPNH} + 12\ H^+ \rightarrow \\ &\qquad 6\ \text{Ribulose-1,5-diphosphate} + 1\ \text{fructose-6-phosphate} + \\ &\qquad\qquad 17\ P_i + 18\ \text{ADP} + 12\ \text{DPN}^+ \end{aligned} \tag{1}$$

It has been estimated that the energy of twenty-four equivalents of photons would be required for the dissociation of the twelve molecules of water and the subsequent DPNH formation which is necessary for the synthesis of one molecule of hexose, but the total energy-transfer process in nature is considerably less efficient than this theoretical value.

The nutritional source of free energy is met in all photosynthetic plants by the preceding system, but differences occur among these plants in the disposition of the hydroxyl complex concurrently formed in the photolytic process (72):

(*a*) In the green plants, peroxide formation is followed by the regeneration of water and the evolution of oxygen.

(*b*) In the strictly anaerobic green bacteria (Chlorobacteriaceae), oxygen cannot be formed, and a hydrogen donor is necessary for the reduction of hydroxyl. This donor is generally H_2S; water and elemental sulfur are formed in the process. Sometimes other inorganic compounds may serve, as in the oxidation of thiosulfate to sulfate by *Chlorobium thiosulfatophilum* (73).

(*c*) The anaerobic purple sulfur bacteria (Thiorhodaceae) may also oxidize various inorganic sulfur compounds and can even utilize molecular hydrogen as the reducing agent for hydroxyl disposal. In these organisms, moreover, various organic acids may be used as the hydrogen donor, although these bacteria are essentially autotrophic.

(*d*) Finally, among the purple nonsulfur bacteria (Athiorhodaceae), only a few species can oxidize inorganic sulfur derivatives, and hydrogen or organic compounds are the principal reducing agents (*74*). The bacteria require certain growth factors, usually biotin because of organic acid involvement (cf. VI,D,2), and thus are not entirely autotrophic. The family is generally microaerophilic, but may be grown under strictly anaerobic conditions in light. Only those species that can grow in the presence of oxygen can be cultured in darkness. Fatty acids are the best hydrogen donors, and the amount of CO_2 fixed is roughly proportional to the number of methylene groups in the acid. It appears that in some instances the organic hydrogen donor is completely oxidized to CO_2 which is subsequently assimilated, whereas in others, intermediate organic compounds may be formed which are utilized directly.

B. CHEMOSYNTHETIC AUTOTROPHS

Carbon dioxide fixation and reduction may occur in some organisms in the absence of light and of all oxidizable materials other than some reducible inorganic substance. Such organisms are conveniently classified according to the nature of this inorganic energy source, although they may not be so closely related on the basis of conventional taxonomic systems (*75*). Among these chemosynthetic autotrophs, relatively little is known concerning the precise mechanisms by which the energy is transferred from the inorganic substrate into organic molecules. A few basic processes, however, seem common to all the members of this group. A specific enzyme system or electron acceptor must initially be activated by the oxidizable substrate through electron transfer, and this reduced enzyme or carrier is in turn oxidized via the cytochrome system. Molybdoflavoprotein are involved in NO_2 and H_2 oxidation in at least some cases. Sulfite oxidation in the rat liver requires lipoic acid (cf. VI,F,2), and a similar requirement may exist in the sulfur bacteria. Further steps in the reaction are probably quite similar to those seen in photosynthesis. It is probable that there are many diverse pathways of electron transfer between the cytochrome system and phosphoglycerate in the various autotrophs (*76*). Brief mention should be made of the commonest types of autotrophy.

1. *Hydrogen Bacteria*

Members of the genus *Hydrogenomonas* obtain their free energy from the direct reduction of oxygen by hydrogen

$$2\ H_2O + O_2 \rightarrow 2\ H_2O + 112\ \text{kcal.} \tag{2}$$

These organisms are capable of heterotrophic growth, but this appears to be independent of their autotrophy, since hydrogen utilization in the presence of lactate oxidation proceeds just as fast as in its absence. The energy yield for hydrogen oxidation is very high (28 kcal. per gram as against 4.1 kcal. per gram of starch), and the free energy efficiency of these bacteria is 10–20% above that of other autotrophs. Some species may function anaerobically with nitrate acting as a hydrogen acceptor, and the unrelated *Micrococcus denitrificans* carries out real denitrification with H_2. Many other bacteria oxidize hydrogen with a variety of electron acceptors, and only some of them are autotrophic. The photosynthetic purple bacteria, previously discussed, are among these. *Desulfovibrio* is an obligate anaerobe that utilizes sulfate, sulfite, or thiosulfate as the hydrogen acceptor. Carbon dioxide can also be used as the hydrogen acceptor, forming methane (*Methanomonas*) or acetic acid (*Clostridium aceticum*).

2. *Nitrifying Bacteria*

Various of the nitrobacteriaceae may obtain their sole energy from the oxidation of ammonia (e.g., *Nitrosomonas*) or nitrite (e.g., *Nitrobacter*), and may, as in the examples cited, be exceptionally fastidious in this nutritional requirement. Oxygen and CO_2 are reduced, although some species may utilize organic compounds as a carbon source.

3. *Sulfur Bacteria*

The colorless sulfur-oxidizing bacteria are of two general taxonomic types. The filamentous Beggiatoaceae (*Thiothrix, Beggiatoa, Thiospirillopsis, Thioplaca*) are structurally almost identical with the blue-green algae of the family Oscillatoriaceae, and there are sulfur bacteria which parallel each of the members of this family of algae. The second type, the unicellular sulfur-oxidizing bacteria, are for the most part members of the thiobacilli (77). While most living organisms are capable of oxidizing sulfur compounds, these species perform this reaction as their sole source of free energy, utilizing H_2S, S_2O_3, S_4O_6, and S as an energy source, and employing CO_2 and O_2 as hydrogen acceptors. *Thiobacillus denitrificans* utilizes nitrate as a terminal electron acceptor anaerobically; *T. ferrooxidans* may utilize ferrous ions instead of thiosulfate as an electron donor; and *T. thiocyanoxidans* is an obligate autotroph which can utilize thiocyanate as an energy, nitrogen, and carbon source. *Thiobacillus thiooxidans* is notable for its ability to produce and live in a medium containing as much as 5% sulfuric acid (pH = 0). Typical general reactions for the process are

$$H_2S + 0.5\ O_2 \rightarrow S + H_2O + 41.5\ \text{kcal.} \tag{3}$$

$$S + H_2O + 1.5\ O_2 \rightarrow H_2SO_4 + 188\ \text{kcal.} \tag{4}$$

Sulfide is presumably oxidized to sulfur, which is enzymatically condensed with sulfite to form thiosulfate. The latter is then oxidized to tetrathionate and thence through trithionate to sulfite and sulfate, some of the sulfite being recycled for condensation with sulfur. Sulfate activation in animal tissues occurs through the agency of adenosine-5′-phosphosulfate and adenosine-3′-phosphate-5′-phosphosulfate (*78*), and in plant and animal tissues a variety of oxidations and reductions of sulfur compounds occur with the sulfur in organic combination, so that organic intermediates are probably involved in most of the steps involving sulfur-bacteria autotrophy (*79*).

4. *Iron Bacteria*

A number of bacteria that are only moderately well established as autotrophs deposit large amounts of ferric oxide around their cellular structures and are presumed to live on the energy derived from the oxidation of ferrous to ferric ions. The oxidation of one mole of iron

$$4\ \mathrm{FeCO_3O_2} + 6\ \mathrm{H_2O} \rightarrow 4\ \mathrm{Fe(OH)_3} + 4\ \mathrm{CO_2} + 40\ \mathrm{kcal.} \tag{5}$$

would presumably yield enough energy to produce 0.209 gm. of cellular material, and give 106.8 gm. of ferric hydroxide, a ratio of iron sheath to cellular material of about 500:1 (*80, 81*). Typical of these organisms are the unicellular genera *Siderocapsa* and *Gallionella,* and the muticellular *Sphaerotilus, Clonothrix, Leptothrix,* and *Crenothrix.* All the organisms are not obligate autotrophs (*Gallionella* is believed to be) and many can oxidize manganous ions instead of iron and deposit manganic oxide sheaths. Numerous other bacteria, protozoans, and algae deposit iron oxide sheaths incidental to other metabolic activities, without using the iron as an energy source.

5. *Carbon Monoxide Bacteria*

Bacillus oligocarbophilus utilizes carbon monoxide as an electron donor. Various workers have reported that this or similar organisms grow in coccoid and encapsulated form when acetone vapor, acetic acid, formalin, or formic acid are used as the substrate, but in threadlike form when carbon monoxide is the carbon source, so that the species is frequently referred to as *Actinomyces oligocarbophilus.* The nutritional source of free energy thus influences the gross morphology. A pseudomonad (perhaps *Hydrogenomonas* sp.) grows in $\mathrm{CO:O_2}$ mixtures containing as high as 80% CO (*82*), according to the general reaction

$$\mathrm{CO} + 0.5\ \mathrm{O_2} \rightarrow \mathrm{CO_2} + 66\ \mathrm{kcal.} \tag{6}$$

The extreme sensitivity of the cytochrome oxidase system to CO makes the energy transfer in these species of particular interest.

6. *Bacteria Oxidizing Methane and Other Hydrocarbons*

Methanomonas methanica oxidizes methane as its sole carbon and energy source according to Eq. 7. Many other bacteria (and fungi) may

$$CH_4 + 2\ O_2 \rightarrow CO_2 + 2\ H_2O + 195\ \text{kcal.} \tag{7}$$

also utilize methane, and various species are known which can use one or more of all of the known aliphatic and aromatic hydrocarbons as a sole source of energy. Most can use more complex organic materials, however, and there has been extensive study of the subject of microbial hydrocarbon oxidation (*34*). A few species (or perhaps "strains") are quite fastidious, utilizing, for example, only methane, or ethane; these have been employed as assay organisms in petroleum exploration. Some anaerobic species may utilize nitrate or sulfate as electron acceptors, but insufficient energy is available for bacterial growth from the methane-sulfate system; indeed it can be shown thermodynamically, and has been demonstrated experimentally, that anaerobic sulfate-reducing bacteria cannot oxidize hydrocarbons smaller than decane (*83*). Higher organisms may also have some ability to utilize hydrocarbons as an energy source, although the subject has been but little studied. *n*-Hexadecane, when fed to rats, is oxidized to fatty acids in the liver to the extent of about 15% of the hydrocarbon fed (*84*), and a variety of evidence in addition to this indicates that mammals, at least, are able to absorb considerable quantities of hydrocarbons from the digestive tract.

C. Heterotrophic Nutrition

Etiolated plants, some algae, many bacteria and fungi, most protozoa, and all known higher animals require compounds more complex than those previously mentioned as a nutritional source of free energy. Most, if not all, aliphatic compounds, including their many derivatives, may be utilized as an energy source by living things, when structural details do not make the compounds prohibitively toxic. A similar consideration holds true for aromatic compounds. The lower plants are the least fastidious in regard to this nutritional need, and there is no doubt that all heterotrophic organisms do best with those compounds that are the most closely related to amino acids, lipids, and carbohydrates. Specific compounds among these, however, are seldom required by organisms as an energy source, with only a few possible exceptions. The honeybee may have specific carbohydrate requirements, and some parasites may also be this fastidious. It appears, for instance, that tapeworms have a chemical need for carbohydrate and are capable of utilizing only certain monosaccharides (*85*). The energy in all the diverse organic substances utilized must be converted biologically to the pond energies of glucose, or intermediates in the glycolytic process, for its ultimate utilization. Were living things incapable of this task, the biosphere would eventually accumulate such vast quantities of organic chemicals as seriously to disturb the carbon cycle in nature, and the earth would become a vast and lifeless garbage heap.

In human nutrition, carbohydrate, protein, and fat supply about 4, 4, and 9 kcal. per gram, respectively; these values may vary somewhat in other species depending upon losses by digestion, absorption, and excretion and upon the specific nature of the diet. Complete replacement of carbohydrate by protein or fat may produce low values due to incomplete oxidation. Among most plants and animals there is a great deal of species specificity with regard to the energy sources that can be utilized. Adult honeybees, for instance, were found in one study to utilize some seven sugars that are sweet to the bee's taste and six others that were tasteless, but not five sugars that were also tasteless (*86*). Many protozoa (and higher animals up to and including mammals) may use acetate for at least a part of their requirement. *Tetrahymena geleii* can utilize glucose, fructose, mannose, and maltose, but not many other sugars including sucrose (*87*). Selectiveness in these instances reflects both intracellular and digestive enzyme patterns in the various species. Differential fastidiousness with regard to nutritional utilization of various carbohydrates is a major parameter in determinative microbiology. Many lower plants and animals contain cellulases. Certain higher animals have also evolved cellulases, chitinases, etc.; the snail *Helix* is the most versatile in this regard; but no vertebrate that has been studied contains β-glucosidase. Cellulases have been found in some earthworms and in the digestive tract of the wood-boring bivalve *Teredo*. In *Teredo*, wood loses some 80% of its cellulose and 15–50% of its hemicellulose during digestion (*88*).

Wood insects are of several types (*89*); some use only the stored starches and sugars in wood; others can digest the cell contents and hemicelluloses; still others have a cellulase of their own; a fourth group contains symbionts that supply the needed cellulase.

The quantitative free energy requirements for metabolic activity are reflected in aerobic organisms by their rate of oxygen consumption (or heat production, or many other functions (cf. I,D) (*90*). At any given temperature, the smaller an animal, the greater will be its energy requirement per unit of body weight. Among small animals, for instance, the long-tailed shrew (weight = 3.4 gm.) has an oxygen consumption of

13.7 ml./gm./hr., as contrasted with a 350-gm. white rat with an oxygen consumption of only 0.77 ml./gm./hr. The food intake of the shrew is at least equal to its own body weight every 24 hours. When values for a large series of different animal species are plotted, it can be seen that the resulting asymptotic curve limits the lower possible weight for mammals to about 2.5 gm., since below this weight an animal would be unable to get enough food for its practically infinite metabolic rate (*91*). Indeed the rate for the shrew is approximately twice that for paramecia. Comparison of energy requirements on a basis of nitrogen content shows good agreement within individual phylogenetic groups and indicates that phylogenetically advanced groups tend to have a higher requirement than more primitive ones (*92*). Among the tissues of a given species the energy requirements vary with the grossly obvious intensity of tissue functions. As measured by the Q_{O_2} milliliters O_2 consumed per milligram tissue per hour), the value for rat retina is 31, for kidney 21, for skeletal muscle 3, and for skin 0.8. Similar considerations hold for plant tissues; the O_2 absorbed (mm.^{3}/gm./hr.) by wheat root tips is 1338, for rootlets in the growth zone 1200, for leaves 1006, and for rootlets in the root hair zone 300–600.

V. Nutritional Requirements for Structural Organization

One of the distinctive properties of life is that it is contained in some specific shape or form. Even the most primitive slime mold or ameba has structural organization within its protoplasm. Cells of other simple organisms have distinctive morphological characteristics, and in the higher plants and animals these cells are organized into tissues with precise characteristics of their own. Finally, these tissues are systematically compounded to create complex living things with distinctive appearances unique to the various species. In order to provide this form and a variable degree of rigidity to these structures, it is essential that certain of the substances of which the organism is composed be less soluble in the fluid medium, provide a certain amount of molecular or crystalline continuity, and be less susceptible to the vicissitudes of continuous metabolic fluctuations. It is also essential that materials be provided to maintain the property viscosity of the intracellular and extracellular fluids and to hold the cells and tissues of complex organisms together in a proper relationship. From a cytological standpoint, it is necessary that structures be provided to maintain the proper functional relationships between the different parts of the cell (e.g., the nuclear membrane), and between the cell and its environment (the plasma membrane). Finally, structures must be provided for recording exact information regarding every detail of that cell type (nucleoproteins), so that this historical record may be used in future duplication of that particular organism.

The chemical substances that perform these needs are generally either very high molecular weight organic compounds (polymers) or relatively insoluble inorganic crystallites. None is required nutritionally, but in many species there is a definite chemical need for the simpler, more soluble moieties of which they are composed. Some of the primary components, such as the amino acids, may have functions other than as structural building blocks, but in relation to their quantitative requirement in the diet, this is their primary function. In terms of chemical needs, the structural substances may conveniently be classified as (*a*) proteins, (*b*) polysaccharides, (*c*) nucleic acids, (*d*) lipids, and (*e*) mineral deposits. It is apparent that certain of the constituents of these materials must be considered from other aspects in separate sections of this chapter.

A. PROTEINS

The structural proteins are composed for the most part of all or most of some twenty different, generally occurring α-amino acids. Several other very rare amino acids may occur in proteins in isolated instances, and some unnatural amino acid analogs have been biologically incorporated into protein structures (*93*). The total amino acid (or protein level) of the diet varies with a number of factors, but for most vertebrates it is about 20%. Fish apparently have much higher requirements; for the Chinook salmon at 47°F. the optimal protein level is 40% of the diet, and at 58°F. it is 55% (*94*). It is also high for insects.

1. *Glycine*

Glycine is synthesized from serine by the action of serine hydroxymethylase (or aldolase), and the complete conversion requires DPN, TPN, GSH, Mn^{++}, pyridoxal phosphate, and tetrahydrofolic acid. Its immediacy to glycolytic intermediate precursors (3-phosphoglyceric acid) results in its absence as a requirement for most species. A few bacteria and protozoa require it, as do mosquito larvae and cultures from monkey tissues. There is a high requirement for the birds that have been studied. The chick has a limited ability to synthesize glycine, and this is stimulated by ethanolamine which appears to be a precursor, but not by its methyl derivatives or by serine. The requirements of 1.5% in the diet of chicks and poults appears in part to be associated with collagen synthesis and with rapid feathering, feather protein containing large amounts of glycine and arginine, for which a similarily high requirement exists (*95*). The major function of glycine is as a constituent of structural proteins in all species, and this is manifest in its high content in collagens, keratins, silk, and invertebrate skeletal proteins. It is also important as a precursor of serine, of various conjugated forms of glycine (glutathione, creatine, sarcosine, hippuric acid, etc.), and of porphyrins through condensation with succinate to form α-amino-β-ketoadipic acid which is converted to porphobilinogen. Tissue demands for glycine are thus high, and interference with its synthesis (e.g., in folic acid deficiency) may produce severe symptoms in a number of otherwise unrelated systems.

2. *Alanine*

Alanine is formed for the most part by direct transamination of pyruvic acid, and its nutritional requirement seems to be limited to a few protists and to the cockroach (*96*) (which should be reinvestigated).

3. *Aspartic and Glutamic Acids*

These acids are formed largely by the direct transamination of oxalacetic and ketoglutaric acids, and their requirement is apparently limited to the Protista. These and the previous two amino acids serve as a major source of amino groups for synthesis of the other "nonessential" amino acids. Aspartic acid contributes its amino group to citrulline in the conversion of the latter to arginosuccinic acid and arginine in the urea cycle. Glutamic acid is a precursor of various conjugates such as glutathione, folic acid, and bacterial capsular substance (a polypeptide containing about 400 D-glutamic acid residues (*97*). Glutamic acid is a precursor of the carbon chain of arginine, ornithine, γ-aminobutyric acid, proline, and hydroxyproline, whereas aspartate is involved in purine and lysine biosynthesis.

Glutamine and asparagine exist in many proteins, and their amide nitrogen is an important source of amino groups for many of the metabolic functions of glutamic acid. They are important storage forms of nitrogen in plants, and asparagine was probably the first amino acid isolated (1806). These amides are formed by glutamine and asparagine synthetase systems in all species studied, but these systems are not active in all animal tissues. As a consequence, glutamine is a nutritional requirement for mammalian (but not chick) tissue cultures, and high levels of glutamic acid cannot substitute for it in mouse L strain cultures, although they can in human HeLa strain cultures. Asparagine is also required for the Walker 256 rat carcinoma cultures, and in this case both amides are required and there is no substituting for either. Glutamic and aspartic acids may be *beneficial* for many rapidly growing species in which they are not required as nutrients, and they are active in certain *Neurospora* mutants (*98*) in which transamination appears to be limited.

4. *Serine*

Serine is synthesized from 3-phosphoglyceric acid, and because of this close relationship to the glycolytic chain it is required in the nutrition of only a very few species (protists, some insects, rabbit tissue cultures). It is required by the male cockroach, but not the female. Aside from its function in glycine synthesis, serine is a precursor of cysteine by way of cystathione formation. Serine also yields phosphatidylserine and the ethanolamine and choline necessary for cephalins and lecithins.

Serine is a precursor of the side chain of tryptophan. A number of serine-requiring mutants of *Neurospora* are known, some of which also respond to glycine and some of which respond to formate, formaldehyde, glycolic acid, and glyoxylic acid. Serratamic acid (D-3-hydroxydecanoyl-L-serine) is found in a *Serratia* species, and *O*-carbamyl-D-serine in *Streptomyces*. Cycloserine (oxamycin) is an antibiotic, as is azaserine, which inhibits purine synthesis (and tumor growth) by interfering with glutamine utilization. D-Serine is the first D-amino acid to be discovered in animal tissues and occurs in the form of lombricine (guanidinoethyl D-seryl phosphate).

5. *Threonine*

Threonine is required by a few protists and by all the animals studied to date. It is the last of the nutritionally important amino acids to be discovered (1935). The requirement for higher animals is about 0.5% of the diet, about 0.5 gm. per day for humans. The lack of a demonstrated requirement in the cockroach may be due to the experimentally troublesome presence of intracellular symbionts detectable with difficulty in this species (*99*). Threonine is required by the trypanosome *Crithidia fasciculata* only when the purine requirement is supplied in the form of the free bases (*100*), and it is said to be replaceable by a tenfold lesser weight of α-aminobutyric acid. Threonineless-*Neurospora* mutants are known which can also utilize α-aminobutyric acid (as does *Crithidia*), or isoleucine. The D-form, the keto derivative, and allothreonine are nutritionally inactive; threonine (like lysine) is unable to derive its amino nitrogen from other amino acids through transamination. Threonine is synthesized in the protists (*a*) from aspartic acid via homoserine or (*b*) by condensation of acetaldehyde with glycine; in both cases the amino group is supplied *with* the carbon chain of another amino acid since ultimate transamination cannot occur. Threonine is a source of glycine in mammals, the liver containing a threonine aldolase which reverses the glycine plus acetaldehyde synthesis of lower plants. The equilibrium is such, however, that threonine cannot be synthesized in this fashion in mammals. The two-carbon fragment (acetaldehyde) which results in mammals may be converted to acetyl coenzyme A (CoA). Liver also contains a threonine dehydrase which forms α-ketobutyric acid; an analogous system in *Crithidia* and *Neurospora* explains their utilization of α-aminobutyric acid. Threonine may be converted to glycogen via glycine, acetyl CoA, or α-ketobutyrate. It is not decarboxylated in animals, but it is by *Streptomyces griseus,* in which it supplies the aminopropanol moiety necessary for vitamin B_{12} synthesis. Threonine is a lipotropic agent in higher animals, and none of the other amino acids can replace it in preventing liver-fat accumulation.

6. *Valine, Leucine, and Isoleucine*

These amino acids are required by some lower plants and by all animal species studied to date. Their biosyntheses are through the corresponding keto acids, but formation of the carbon chains involves complex processes which are incompletely understood. Valine originates through pyruvate and acetyl CoA condensation to form acetolactate; subsequent methyl group migration produces α-ketoisovalerate which transaminates to valine. The latter keto acid also condenses with acetyl CoA to yield a product which leads through a series of dicarboxylate intermediates (resembling the tricarboxylates of the citrate cycle) to isocaprolate and by transamination to leucine. Isoleucine originates in α-ketobutyrate and pyruvate, and by steps similar to those in valine synthesis, arrives at its α-keto acid. D-Forms are inactive for *Tetrahymena* but may show some activity in the rat. The corresponding hydroxy and keto acids are also inactive in *Tetrahymena,* but the keto acids are generally active in mammals. The requirement for valine or isoleucine in mammals is about 0.6% of the diet; for leucine it is about twice this. These same relative proportions occur in most proteins. Leucine is a remote precursor of the isoprene unit of carotenoids and steroids, the pathway leading through mevalonic acid to farnesyl pyrophosphate and squalene. The utilization of leucine in carotene synthesis has been extensively studied in *Phycomyces blakesleeanus* (*101*).

7. *Cystine and Cysteine*

Cystine is not required by most animals because of its ready synthesis from methionine and serine, but it is essential in cell cultures because of a block in methionine demethylation. It may be required, however, when the nutritional supply of methionine is sufficiently low to limit its synthesis. Cystine is essential for the normal formation of puparia and for metamorphosis to the adult stage of *Lucilia sericata* and *Drosophila.* The absence of cystine, even in the presence of large amounts of methionine, causes reduced growth and survival of mosquito larvae and a high mortality rate in adults formed from the pupae. Glutathione (cf. VI,C,7), a product of cystine, permits normal emergence, but does not improve growth rate or survival. In the absence of cystine from the cockroach diet, there is similarly a high mortality rate, particularly during ecdysis (*102*). Mutants of *Glomerella* have been obtained which require glutathione, can utilize a mixture of glutamylcysteine and cysteinylglycine, but are blocked in dipeptide formation and so cannot utilize the three free amino acids. Cystine is an important intermediate in inorganic-organic sulfur transformation in all forms of life, and the cystine-cysteine redox system is a universal one in biology. Cystine is a precursor of biotin, of virtually all urinary sulfates, and of taurine and a variety of less common sulfur metabolites.

8. *Methionine*

Methionine is apparently required by all the animals studied to date, with the reported exception of *Phormia regina,* which does not require it in the presence of adequate levels of cystine (*103*). Its D-form is generally active. In higher animals it is required to the extent of 0.3–0.5% of the diet. Neither methionine, nor its carbon skeleton, nor homocysteine which can be methylated to form it, can be synthesized by the animals which have been studied. It is formed in a mutant of *Aerobacter aerogenes* by the transmethiolation of α-aminobutyric acid with thiomethyladenosine, but this biosynthetic scheme may not be general. Under some circumstances, small amounts of methionine may be synthesized by animals from homocysteine. Methionine participates in transaminations readily, so that its keto acid (α-keto-γ-methiolbutyric acid) is nutritionally active. Methionine functions as a source of cyst(e)ine, of methyl groups, of α-ketobutyric acid, and of metabolic sulfur. Its role in transmethylation through formation of *S*-adenosylmethionine is critical in the synthesis of a great number of metabolites, including the porphyrin-like moiety of vitamin B_{12} (*104*).

9. *Proline and Hydroxyproline*

These acids are derived from glutamic acid by the rather direct cyclization of its semialdehyde, so that proline is required in only a very few animal organisms, and its oxidation product, hydroxyproline, is not required by any plant or animal studied to date. The occurrence of hydroxyproline in nature is almost entirely limited to certain animal structural proteins, such as collagen, and there is little replacement of collagen hydroxyproline by administered hydroxyproline. While it is unlikely that collagen proline is hydroxylated during collagen synthesis, the nature of the precursor of collagen hydroxyproline remains to be identified (*105*). Proline, hydroxyproline, and ketoproline have all been identified in the antibiotic, actinomycin I (*106*), which is synthesized by *Streptomyces antibioticus,* so that it is possible that these imino acids play an as yet unidentified role in at least the lower members of the plant kingdom.

10. *Hydroxylysine*

This compound, derived by the oxidation of lysine, is similar to hydroxyproline in that it is not known to be a nutritional requirement; its distribution is limited to some collagens and collagen-like materials, and administered hydroxylysine is not appreciably incorporated into tissue collagen (*107*).

11. *Arginine*

Arginine is required by some animals and a few lower plants, but many can dispense with it since its synthesis is rather directly from glutamic acid via ornithine. It is not required in man, but it is in most other vertebrates and in the insects studied to date. It is an absolute requirement in mammalian cell cultures. The D-form is generally active, and citrulline will replace at least a portion of it. Citrulline has very limited activity for the Protozoa, and ornithine is generally inactive in most animals and lower plants. *Neurospora* mutants have been isolated which will respond to: (*a*) only arginine; (*b*) arginine or citrulline; and (*c*) arginine, citrulline, or ornithine. Many of the existing data are undoubtedly misleading because of the differing effects which may be produced among various species by slow arginine synthesis. An indispensable dietary component has been defined (*108*) as "one which cannot be synthesized by the animal organism, out of materials ordinarily available, at a speed commensurate with the demands for normal growth." It is in the case of arginine, more than any other amino acid, that the rate of synthesis may be limiting, and that the demands for normal growth may be a questionable parameter for the assessment of chemical needs. Arginine accelerates the growth of rats (and probably that of other animals), but it is apparently not necessary for growth or maintenance of nitrogen balance in adult mammals. The requirement for growing mammals is generally set at about 0.25% of the diet. The requirement for birds is notably high, having been assessed for young chicks at about 1.2% of the diet, and 1.6% of the diet for poults. It is also high in the guinea pig. A major function of arginine is in the production of urea via the Krebs-Henseleit cycle. It is even more generally important as a precursor of the phosphagens, phosphocreatine, and phosphoarginine, and this latter fact may explain its reported requirement by virtually all the invertebrates studied to date. Arginine is present in large amounts in the protamines and histones and is quite low in the glutelins. Arginine is probably the precursor of spermine, a factor required for the growth of some bacteria. The protamines frequently contain over 50% arginine, and their high concentration in sperm, along with the known function of spermine in seminal fluid and the suppression of spermatogenesis in human arginine deficiency, suggests a special function for arginine in the metabolism of the spermatozoa (*109*).

12. *Lysine*

Lysine is required by all animals and some lower plants. Like threonine, its carbon chain is not synthesized by animals, so that its α-amino group is not derived from or susceptible to transamination. As a result, the D-form is nutritionally inactive. It is synthesized by two different routes of phylogenetic interest (cf. I,C), the one starting with aspartic acid (in bacteria), and the other with glutamic acid (yeasts and molds). Only small amounts are required in the diet of adult animals for maintenance of nitrogen balance, probably because of lysine's refractoriness toward transamination. For most higher animals the requirement is about 1% of the diet, although this is somewhat higher in poults because of the presence in their feathers of a high-lysine, melanin-like pigment (*110*). Synthesis of lysine may be vigorous in lower animals, some fungi producing up to 20 gm. per liter of medium (*111*). The cell walls of penicillin-sensitive gram-positive bacteria are built of a lysine-containing, peptide-amino sugar polysaccharide, but in some cases the lysine precursor, α,ϵ-diaminopimelic acid, is present in the peptide instead of lysine (*112*). Biotin frequently occurs in nature as ϵ-*N*-biotinyllysine. These and other facts suggest that lysine may have a number of vital roles in metabolism that have not as yet been elucidated.

13. *Histidine*

Histidine is required in the nutrition of some bacteria and phytoflagellates and by all animals studied. It is reported to be nonessential for maintenance of the nitrogen balance of adult humans, but it seems questionable that tissue synthesis occurs in this instance. It is required by human cell cultures. Requirements are much lower for all adult animals than they are for the young, and limited intestinal synthesis may occur. The D-form and the hydroxy and keto forms are generally active, but D-histidine is inactive in humans. Imidazole acrylic acid and imidazole itself are inactive. Requirements are generally in the range of 0.3% of the diet in young animals. Histidine is synthesized from adenosine-5′-monophosphate, ribose-5-phosphate, and glutamine. The purine nucleotide plays a catalytic role in that, although a part of it is utilized for imidazole synthesis, it is subsequently regenerated. Since the precursors of histidine are all present in animal tissues, there appears to be no fundamental reason why some animal tissue could not synthesize this amino acid. The genetic aspects of the biosynthetic sequence have been extensively studied in *Salmonella* through the investigation of over 200 auxotrophs (*113*). Histidine is decomposed by animal tissues to yield glutamic and formic acids. Other common products of mammalian histidine metabolism include imidazole acetic acid (which is excreted as a riboside), histamine, carnosine, and anserine. Ergothioneine, the betaine of thiolhistidine, is present in high concentrations in mammalian erythrocytes, liver, brain, and boar semen. It is of dietary origin, however, and its concentration in certain tissues is unexplained. Synthesis of ergothioneine is apparently limited to microorganisms; high levels in plant seeds are due to attendant fungi.

14. *Phenylalanine*

This amino acid is required by all animals studied, with the single reported exception of the cockroach (*114*). The keto form is generally active; the D-form is active in the rat, mouse, and flour beetle, but not in man and many other species. Animals are unable to synthesize the phenylnucleus, so that phenylalanine and tryptophan provide the major nutritional sources of this aromatic group. The phenylalanine requirement is generally about 0.7% of the diet, but this must be increased to about 1% if tyrosine is absent. The amino acid is synthesized from glucose via shikimic acid and phenyl pyruvate in the microorganisms that have been studied. Other than as a protein constituent, the major function of phenylalanine is as a precursor of tyrosine.

15. *Tyrosine*

Tyrosine is formed by the direct oxidation of phenylalanine in most animals (*115*), and it is thus not required in the diet when ample amounts of phenylalanine are present. This fact has resulted in many misleading statements in the literature regarding the tyrosine requirement. Tyrosine is, however, an absolute requirement for mammalian cell cultures. Tyrosine cannot be converted back into phenylalanine in animals. Aside from its function as a protein constituent, tyrosine (and thus phenylalanine) is a precursor of numerous biologically important substances (melanin, epinephrine, diiodotyrosine, thyroxine, dibromotyrosine, coumarins, lignins, etc.) (*105*). Nutritional or genetically induced deficiencies of phenylalanine metabolites may result in a variety of pigmentation defects, such as albinism (lack of skin pigment) and alkaptonuria (urinary pigmentation). These have been demonstrated in insects as well as mammals. Phenylketonuria is a human congenital defect in the ability to convert phenylalanine to tyrosine. It is associated with mental deficiency, is inherited as a recessive Mendelian characteristic, and occurs in about 1 in 200 individuals. Restriction of the phenylalanine intake in this condition lowers the blood level of this amino acid to normal, abolishes the excretion of abnormal metabolites, and lessens the severity of the mental defect. Since the mental improvement appears to be correlated with the earliness at which the defect is detected, testing of the urine of all newborn for phenylpyruvic acid has been recommended; abnormal metabolites of the accumulated phenylalanine have been suggested as etiologic factors in the mental deficiency.

16. *Tryptophan*

Tryptophan is required by all animals studied to date. The D-form and the keto form are active in some animals, but not others. The requirement is generally about 0.2% of the diet. In the guinea pig, optimum growth is obtained with 0.13% in the diet, but ocular defects are not prevented at levels below 0.16%. In this instance, the requirement for one specific organ (the eye) exceeds that for optimum growth (*116*), emphasizing again the dangers associated with using optimum growth alone as a criterion of nutritional adequacy. In plants tryptophan is synthesized from shikimic acid via anthranilic acid, and subsequently indole plus serine. Two pathways are involved in converting anthranilic acid to indole, the one (in *Saccharomyces*) involving condensation with fructose-6-phosphate, and the other (in *Escherichia coli*, *Neurospora*, and *Salmonella*) employing condensation with 5-phosphoribosyl-1-pyrophosphate. Indole is nutritionally active in supplying the need for tryptophan in some of the bacteria and fungi which require tryptophan (most do not), and some of the indole-utilizing species may also utilize anthranilic acid (*117*). The nutritional interrelationship between tryptophan and phenylalanine in plants is undoubtedly due to the presence of a common aromatic precursor for both amino acids (*118*). Tryptophan is the precursor of a number of other important biological substances. Among these are numerous indole alkaloids, including the highly active ergot group, and indoleacetic acid (heteroauxin). About 3% of dietary tryptophan is metabolized to 5-hydroxytryptophan derivatives, such as 5-hydroxyindoleacetic acid. In malignant carcinoid as much as 400 mg. per day of this metabolite may be excreted in the urine (normal value about 7 mg. per day), a considerable loss in view of the tryptophan intake. Other 5-hydroxy derivatives include serotonin, a probable neurohumoral agent in most animals and a constituent of many insect, amphibian, and cephalopod venoms. Rupture of the indole ring of tryptophan yields formylkynurenine, a precursor of insect eye pigments (ommochromes) (*119*). Formylkynurenine is also a precursor of niacin in most plants and animals, but not in the cat (*120*) or in some higher plants such as corn and tobacco (*121*).

17. *Conclusions*

From the accompanying summary of amino acid requirements (Table II), certain general conclusions may be drawn regarding their distribution. There is virtually no nutritional requirement for amino acids among the higher plants; within the protista there is a great diversity of requirements; and among the animals there is remarkable uniformity. The probability of any specific amino acid being required is roughly a function of the number of biosynthetic steps that are required between it and glycolytic precursors, i.e., the number of genetic loci that may be available to undergo mutation. Biosynthetic sequences are quite uniform

TABLE II

Amino Acid Requirements[a]

Amino acid	Plants				
	Autotrophs	Primitive heterotrophs	Phytoflagellates		
	Photosynthetic and chemosynthetic	Most yeasts and molds	*Chlamydomonas chlamydogama* (chlorophyte)	*Gyrodinium cohnii* (dinophyte)	*Hemiselmis virescens* (crytophyte)
Glycine	-	-	-	-	-
Alanine	-	-	-	-	-
Aspartic acid	-	-	+	-	-
Glutamic acid	-	-	-	-	-
Serine	-	-	-	-	-
Threonine	-	-	-	-	-
Valine	-	-	-	-	-
Leucine	-	-	-	-	-
Isoleucine	-	-			-
Cyst(e)ine	-	-			-
Methionine	-	-	-	-	-
Proline	-	-	-	-	-
Arginine	-	-	-	-	-
Lysine	-	-	-	-	-
Histidine	-	-	[illegible]	-	-
Phenylalanine	-	-	-	-	-
Tyrosine		-	-		-
Tryptophan	-	-	-		-

[a] +, required; -, not required.

TABLE II (*continued*)

Amino Acid Requirements

Amino acid	Plants					
	Heterotrophic bacteria					
	Salmonella typhosa	*Neisseria gonorrhea*	*Acetobacter suboxidans*	*Lactobacillus plantarum*	*Lactobacillus arabinosus*	*Leuconostoc mesenteroides*
Glycine	-	-	-	-	-	+
Alanine	-	-	+	-	-	-
Aspartic acid	-	-	-	-	-	+
Glutamic acid	-	+	-	+	+	+
Serine	-	-	-	-	-	+
Threonine	-	-	-	-	+	+
Valine	-	-	+	+	+	+
Leucine	-	-	-	+	+	+
Isoleucine	-	-	+	+	+	+
Cyst(e)ine	-	+	-	+	+	+
Methionine	-	-	-	-	+	+
Proline	-	-	-	-	-	+
Arginine	-	-	-	-	+	+
Lysine	-	-	-	-	-	+
Histidine	-	+	-	-	-	+
Phenylalanine	-	-	-	-	+	+
Tyrosine	-	-	-	-	+	+
Tryptophan	+	-	-	+	+	+

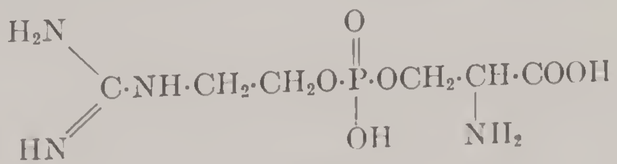
throughout all living things, although alternate pathways do exist in some cases among the simple plants. The relative amounts of the various amino acids that are required by any single species are roughly proportional to the amounts that occur in that species, and this is reflected in the free amino acids in the body fluids, in the total animal proteins, and in the composition of certain proteins unique to the species. The high requirements for glycine and arginine in birds, for instance, are reflected in concentrations of these two amino acids in egg albumin that are five- and twofold higher, respectively, than they are in lactalbumin. It may be estimated that the total protein requirement of a species is defined by the requirement for some one limiting amino acid, since for all vertebrates on which data are available, over 90% of the required protein level is essential to supply the required level of the most limiting amino acid (based on requirements for young animals and on data from egg and milk proteins). The limiting requirement in fish and birds seems to be arginine; both have special needs, and neither can synthesize it (*122*). The limiting requirement in mammals is usually phenylalanine or tryptophan. Very high levels of specific amino acids may be required for the synthesis of specific enzymes.

TABLE II *(continued)*

Amino Acid Requirements

Amino acid	Protozoa			Insects		
	Zooflagellates		Ciliate	Orthoptera	Coleoptera	
	Crithidia fasciculata (trypanosome)	*Trichomonas foetus* (trichomonad)	*Tetrahymena pyriformis*	*Blatella germanica* nymphs (cockroach)	*Attagenus* sp. larvae (carpet beetle)	*Tribolium confusum* (flour beetle)
Glycine	-	+	-	-	-	-
Alanine	-	-	-	+	-	-
Aspartic acid	-	-	-	-	-	-
Glutamic acid	-	-	-	-	-	-
Serine	-	+	-	+	-	-
Threonine	+	+	+	-	+	+
Valine	+	+	+	+	+	+
Leucine	+	+	+	+	+	+
Isoleucine	+	+	+	+	+	+
Cyst(e)ine	-	-	-	+	-	-
Methionine	+	+	+	+	+	+
Proline	-	+	-	+	-	-
Arginine	+	+	+	+	+	+
Lysine	+	+	+	+	+	+
Histidine	+	+	+	+	+	+
Phenylalanine	+	+	+	-	+	+
Tyrosine	+	-	-	-	-	-
Tryptophan	+	+	+	+	+	+

The similarity between the amino acid requirements of the Protozoa on the one hand, and the insects and vertebrates on the other, would seem to discourage hope of finding differences in the many intermediate and unstudied phyla. It is precisely in these intermediate phyla, however, that the greatest diversity of form and function does occur, and that requirements for the "nonessential" amino acids might arise. Moreover, rare varieties of amino acids are continuously being discovered in plant materials and may exist (and be required) in some animal groups. The iodo- and bromotyrosine derivatives, components of the gorgonins and spongins of lower metazoa, are a case in point, as is lombricine (II) in the earthworm (*123*). Hydroxylysine is absent from the cuticular collagen of the nematode *Ascaris* and the annelid *Lumbricus*, but present in the more advanced echinoderm *Holothuria forskali* (*124*). The diffi-



$$\mathrm{(H_2N)(HN{=})C{\cdot}NH{\cdot}CH_2{\cdot}CH_2O{\cdot}\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}}{\cdot}OCH_2{\cdot}\underset{\underset{\displaystyle NH_2}{|}}{CH}{\cdot}COOH}$$

(II)

TABLE II *(continued)*

Amino Acid Requirements

Amino acid	Insects					
	Diptera					
	Musca vicina (housefly)	*Calliphora vicina* (blowfly)	*Phormia regina* (blowfly)	*Drosophila melanogaster* (fruitfly)	*Aedes aegypti* (mosquito)	*Pseudosarcophaga affinis* (spruce budworm parasite)
Glycine	-	-	-	-	-	-
Alanine	-	-	-	-	-	-
Aspartic acid	-	-	-	-	-	-
Glutamic acid	-	-	-	-	-	-
Serine	-	-	-	+	-	-
Threonine	+	+	+	+	+	+
Valine	+	+	+	+	+	+
Leucine	+	+	+	+	+	+
Isoleucine	+	+	+		+	+
Cyst(e)ine	-	+	+	-	+	-
Methionine	+	+	-	+	+	+
Proline	-	-	+	-	-	-
Arginine	+	+	+	+	+	+
Lysine	+	+	+	+	+	+
Histidine	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-
Tryptophan	+	+	+	+	+	+

culties encountered in maintaining the lower invertebrates in axenic cultures (2), let alone a defined medium, may indeed be due to qualitatively different amino acid needs for these animals; indeed, unidentified, low molecular weight growth factors, either protein bound or produced by proteolysis, are required for the growth of mammalian cell suspensions in an otherwise chemically defined culture medium (*125*).

B. Polysaccharides and Lipids

The chemical needs for polysaccharide synthesis have scarcely been investigated. Partly, the deficit in information is conceptual in nature. It is quite apparent that a portion of the carbohydrate available for free energy and as a carbon skeleton for amino acid biosynthesis is used in polysaccharide formation in all organisms, but it is difficult to segregate this portion of the total requirement. In addition, since specific monosaccharides are seldom visualized as nutritional requirements, it seems unlikely that future evidence would reveal such chemical needs. Nevertheless, a rapidly growing body of evidence indicates that polysaccharide structure may be remarkably complex and that the monosaccharides of

TABLE II *(continued)*

Amino Acid Requirements

Amino acid	Vertebrates							
	Fish	Birds		Mammals				
	Chinook salmon	Chicken	Chicken fibroblast tissue culture	Mouse	Mouse fibroblast tissue culture	Rat	Rat tumor tissue culture	Rabbit fibroblast tissue culture
Glycine	-	+	+	-	-	-	-	-
Alanine	-	-	-	-	-	-	-	-
Aspartic acid	-	-	-	-	-	-	-	-
Glutamic acid	-	-	-	-	-	-	-	-
Serine	-	-	-	-	-	-	-	+
Threonine	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+
Isoleucine	+	+	-	+	+	+	+	+
Cyst(e)ine	-	+	+	-	+	-	+	+
Methionine	+	+	+	+	+	+	+	+
Proline	-	-	-	-	-	-	-	-
Arginine	+	-	+	-	+	-	[illegible]	+
Lysine	+	+	+	+	+	+	+	+
Histidine	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+
Tyrosine	-	-	+	-		-	+	+
Tryptophan	+	+	+	+	+	+	+	+

which they are composed may be considerably more diversified in structure than was once supposed. Examples are common: the lipopolysaccharide endotoxins of salmonella contain four different 3,6-dideoxyaldohexoses (*126*); 1-*O*-β-benzoyl-D-glucose occurs in the solid excretion (periplanetin) on the necks of cockroaches (*127*); and a polysaccharide from *Bacillus subtilis* contains a 2,4-diamino-2,4,6-trideoxyhexose (*128*). It would seem probable that chemical needs would develop in some species for certain of these complex esoteric sugars. In a sense, this happens with the type-specific capsular polysaccharides of the pneumococci, where the formation of capsular material by a rough form injected into a host is controlled by the requirement for type-specific DNA which permits polysaccharide synthesis. Further, common polysaccharide-synthesizing enzymes often appear to *require* a small amount of polysaccharide as a template to initiate synthesis. With regard to the simpler polysaccharides (starches, glycogens, celluloses, chitins, pectins, dextrans, etc.), little can be said other than that some quantitative requirement exists for their synthesis. The requirements for chitin in

TABLE II *(continued)*

Amino Acid Requirements

Amino acid	Vertebrates				
	Mammals				
	Dog	Pig	Monkey kidney or testicle tissue culture	Human	Normal human uterine fibroblasts or cervical carcinoma tissue culture
Glycine	-	-	+	-	-
Alanine	-	-	-	-	-
Aspartic acid	-	-	-	-	-
Glutamic acid	-	-	-	-	-
Serine	-	-	-	-	-
Threonine	+	+	+	+	+
Valine	+	+	[illegible]	+	+
Leucine	+	+	+	+	+
Isoleucine	+	+	+	+	+
Cyst(e)ine	-	-	+	-	+
Methionine	+	+	+	+	+
Proline	-	-	-	-	-
Arginine	-	-	+	-	+
Lysine	+	+	+	+	+
Histidine	+	+	-	-	+
Phenylalanine	+	+	+	+	+
Tyrosine	-	-	-	+	+
Tryptophan	+	-	+	+	+

arthropods may be more demanding, as might well be the needs for formation of the cellulose-like coat (tunicin) which covers the epidermis of tunicates and is an exclusive characteristic of the urochordates. The requirements for nitrogen, phosphorus, and sulfur are obviously increased by the formation of some polysaccharides, especially the mucoproteins or aminopolysaccharides, and the glycolipids or lipopolysaccharides. Only in the case of the slime molds has the structural requirement for carbohydrate been definitely evaluated, for in this case glucose is not primarily a carbon or energy source, and in appropriate media it affects neither the rate nor amount of growth (*129*). Its deficiency seriously inhibits the fruiting process in Dictyosteliaceae, however, presumably by preventing synthesis of the polysaccharide which binds together the cells and supports the fruiting body.

Chemical requirements for lipid, lipoprotein, and liposaccharide synthesis are unknown (steroid and "essential fatty acid" requirements are discussed in VI,I and VI,C,5). Lecithin and cephalin synthesis obviously implies a considerably increased demand for phosphate, and for ethanolamine, serine, and choline in organisms requiring these moieties in the diet. Similar considerations hold for the sphingomyelins, although sphingosine has not been reported as a nutritional requirement; inositides and cerebrosides would of course require inositol and galactose.

C. Polynucleotides

The purines and pyrimidines which make up the nucleotides, and the nucleotides which constitute DNA, RNA, and the nucleoproteins, have many functions other than those associated with structure. Nevertheless, assembly of these substances into precisely ordered polymeric structures is fundamental to all of biology, so that the chemical need for these materials for catalyst-carrier functions is also included here only as a matter of convenience. The free bases may function as precursors of other metabolites; for example, in the conversion of purine to histidine (cf. V,A,13) or of pyrimidine to thiamine. The free bases also occur as such in nature, and are known to have special functions in some instances. The postretinal reflecting surface (tapetum lucidum) of the eyes of many arthropods, fishes, amphibia, and reptiles is composed of rather large guanine crystals [a function performed by crystals of a zinc-cysteine complex in carnivores, and by riboflavin crystals in the tapetum of the lemur *Galago* (*130*)]. The nucleotides may function in the transport of high-energy phosphate bonds, as in the case of AMP, ADP, and ATP (*131*). They also function in electron transfer (redox) coenzymes such as DPN, TPN, and FAD, along with riboflavin and niacin; and in active acetate transfer as part of coenzyme A. Adenine nucleotides occur in some of the pseudocobalamins. Since these nucleotides function in a great many different enzyme systems in intermediary metabolism, their biological requirement is as fundamental as that of the enzymes themselves. Aside from the commoner purine and pyrimidine bases (uracil, thymine, adenine, guanine, and cytosine), a large number of other methyl and amino derivatives have now been reported to occur in nucleotides (*132*).

The chemical needs of various species for purine and pyrimidine derivatives are unique in that they appear to vary inversely with the degree of phylogenetic development. Requirements are common among plants, occasional in protozoa, rare in insects, and apparently nonexistent in vertebrates.

1. *Plants*

Photosynthetic plants are able to synthesize all the nucleotides that are essential for their metabolic activities, but not all parts of the plant can do so. Plant roots vary in this respect, tomato and flax roots, for instance, being able to synthesize the pyrimidine moiety of thiamine, and pea roots requiring it. The bacteria, yeasts, and molds also vary greatly in their ability to synthesize this important thiamine precursor (*133*). The first demonstration of the nutritional requirement of a nitrogenous base was the demonstration in 1936 of the essentiality of uracil for the anaerobic growth of *Staphylococcus aureus*. Adenine was soon found to stimulate lactic acid production by *Streptococcus lactis,* to stimulate the growth of *Lactobacillus plantarum,* and to be essential for the growth of a strain of hemolytic streptococcus and of *Clostridium tetani*. Similar observations were made with other nitrogenous bases, and it rapidly became apparent that each of the purines and pyrimidines may appear as an absolute requirement, or at least as a stimulatory factor, in some lower plant species. In general, the bases are replaceable by their oxy

TABLE III

Microorganisms Available For The Biological Assay Of Purines And Pyrimidines

Nutritional requirement	Requiring microorganism and A.T.C.C. number[a]
Precursors	
Deoxyribosides	*Lactobacillus acidophilus* 4963 and 11,506
Ureidosuccinic acid	*Lactobacillus bulgaricus* 10,812
Orotic acid[b]	*Lactobacillus bulgaricus* 10,812
Purines	
Adenine	*Neurospora crassa* 11,063
Pyrimidines	
Cytosine	*Lactobacillus brevis* 8287
Cytidine	*Neurospora crassa* 11,993
Thymine	*Escherichia coli* 9723h, *Lactobacillus* sp. 335
Thymidine	*Lactobacillus leichmannii* 7830, *Lactobacillus lactis* 8000, *Leuconostoc mesenteroides* 8293
Uracil	*Lactobacillus brevis* 8287, *Lactobacillus* sp. 335
Uridine	*Neurospora crassa* 11,993

[a] A.T.C.C. = American Type Culture Collection.

[b] Five to ten times as active as its precursor, ureidosuccinic acid.

derivatives (uracil for cytosine, xanthine for guanine). Uracil-4-carboxylic acid (orotic acid) is active for *Lactobacillus casei.* The nucleosides uridine and cytidine and their nucleotides, uridylic and cytidylic acid, are from ten to thirty times as active as are uracil and orotic acid in stimulating the development of certain mutant strains of *Neurospora.* Thymine or thymidine may substitute for folic acid in *Streptococcus faecalis,* but are required in 5000-fold amounts; in this instance they are providing the product of folic acid catalysis, rather than literally "replacing" it. The literature on the nucleoside requirements for microorganisms is now voluminous (*134*). A list of some common ones that are sufficiently specific to be used for assay purposes is provided in Table III. Bacteria are apparently able to incorporate many "unnatural" purines and pyrimidines into nucleic acids (*134*), and the antitumor activity of such analogs is in part due to their similar incorporation into the nucleic acids of neoplastic tissues (*135*). For a similar reason, many such analogs are found to be mutagenic. Various microorganisms may be adapted to require unnatural analogs, although such requirements may again be lost after repeated transfer in diminishing amounts of the unnatural requirement.

2. *Protozoa*

Within the phytoflagellates there is frequently a nutritional requirement for the pyrimidine moiety of thiamine, even in the absence of any other organic requirement. *Acanthamoeba castellani,* a rhizopod, has a similar requirement. Purines and pyrimidines are required for optimum growth of a malarial protozoan, *Plasmodium knowlesii.* Guanosine is required by the trypanosome *Crithidia fasciculata.* Adenosine spares, but does not replace, the guanosine. *Paramecium multimicronucleatum* and *P. aurelia* require cytidylic and guanylic acids as growth factors. The suctorian *Tokophyra infusionum* achieves optimum activity on a mixture of guanylic, adenylic, and cytidylic acids and uracil; the purine bases are without effect. In *Tetrahymena,* guanine is an absolute purine requirement, and uracil, cytidine, or cytidylic acid (but not cytosine) fulfill the pyrimidine requirement (*4, 11*). In *Trichomonas vaginalis,* an antagonism exists between ribonucleosides and ribonucleotides (*100*). RNA, but not DNA, permits growth, and the RNA can be replaced by a mixture of the four ribonucleotides (adenylic, guanylic, uridylic, and cytidylic acids) or the corresponding free bases. The related nucleosides, by contrast, actually inhibit growth in media containing the nucleotides or free bases. *Trypanosoma cruzi* synthesizes its purines, but not at a rate adequate for optimal growth (*136*).

3. *Insects*

Requirements for purines and pyrimidines are rare. *Drosophila melanogaster* strains require adenine, and RNA is needed for the optimum growth of *Aedes aegypti.* RNA is said to be beneficial for various other dipterous larvae and for the beetle *Sciobius granosus* (*137*), but in these cases it is not required. Thus only two instances of a definite requirement can be shown in the score or more of insects that have been studied.

4. *Vertebrates*

No instance of a chemical need for purines, pyrimidines, or their derivatives has been reported in the numerous vertebrates studied to date. This seems even more striking in view of the absence of a reported requirement in any vertebrate tissue culture study. In this latter instance, all the nucleic acids are derived from glucose and amino acids; 40% of the pyrimidine carbon and 50% of the total purine and pyrimidine nitrogen are derived from glutamine alone (*138*).

D. MINERAL DEPOSITS

Specific but poorly defined mineral requirements exist in most phyla for the provision of a skeletal framework. In the higher plants, rigidity is apparently provided by cellulose fibers, but silicates play some role in this regard. Plants such as wheat will grow without silicates, but the stalks are unable to stand up in a breeze and are more susceptible to destruction by parasites than are plants grown in the presence of silicates. Thus silicon, while not essential, serves an important function, and in this regard it is analogous to fluoride in the higher animals. In green plants, silicon apparently functions also in some aspect of iron transport. The organic matrix of the higher plant skeleton is at best but lightly impregnated with mineral materials.

Mineralized deposits occur in association with the cell wall of many algae, bacteria, and fungi, but in most cases these deposits are an incidental consequence of other cellular activities. In the Protozoa, however, highly organized skeletal structures are common. Among the phytoflagellates are found the calcareous skeletons of the Coccolithophoridae and the siliceous skeletons or latticework of the Silicoflagellata. The Sarcodina are represented by the foraminifera, in which calcareous skeletons commonly occur. Siliceous skeletons are also seen in such species as *Euglypha.* Skeletons are composed of sand grains and other foreign bodies embedded in a nitrogenous matrix in some species. Other amebas are represented by the Radiolaria, in most cases with their endoskeletal siliceous lattice, and a strontium sulfate skeleton in Acantharia. A number of ciliate protozoans also have calcareous skeletons. In the Protozoa, as in the higher animals, mineralized skeletal deposits always have an organic matrix; the degree of mineralization of this matrix may vary considerably among different species.

The nutritional requirements for siliceous skeleton formation, and the metabolic processes involved in its formation, are unknown. The tests and shells of calcareous protozoa are composed almost entirely of calcium carbonate, and hydroxyapatite (complex calcium carbonate phosphate) is generally regarded as unique to the vertebrates. Certain unicellular algae (*Polytoma uvella, Polytomella caeca, Chlorogonium elongatum*), however, deposit a hydroxyapatite-like material in their flagella when grown in media made up with tap water, but not with distilled water. The ciliate *Spirostomum ambiguum* has also been shown to deposit large amounts of hydroxyapatite-like material within the cytoplasm of older cultures (*139*).

Different species of sponges possess skeletal structures of either calcium carbonate or silica, and some corals contain calcium phosphate in their calcareous skeleton.

Calcium carbonate constitutes 87–97% of the shell of mollusks, water and organic conchiolin accounting for the remainder. Constant calcium exchange occurs between the shell and the medium, accounting for about 1% of the calcium deposited by oysters. Shell formation in this case may be studied *in vitro*, and a number of details are known regarding the calcification process (*140*).

In echinoderms such as the sand dollar (*Echinarachnius parma*) addition of lithium to the sea water medium results in skeletal abnormalities. Withdrawal of calcium or of sulfate causes failure of the skeleton to develop (*141*). The minimum calcium requirement necessary for larval development as a whole is often found to be higher than that required for continued skeletal development (*142*). The withdrawal of calcium from sea water to levels one-fifth that of normal (to 80 mg. per liter) produces marked inhibition of skeletal formation, so that the calcium requirement exceeds this level by a considerable amount.

The percentage of calcium phosphate in the mineral material of crustacean exoskeletons is much higher than that found in the lower invertebrates and approaches the level in the vertebrates. In the shrimp *Squilla* it is 17.7%, in *Chloridella empusa* it is 50%, and in *Temore longicornis* and *Thysanoessa inermis* it is 90% and 92%, respectively (*139*). Molting is a complex process in crustaceans, and in the premolt stage calcium is withdrawn from the exoskeleton and stored in the gastroliths or hepatopancreas. After the molt is complete, calcium is again mobilized and redeposited in the skeleton. Adequate amounts for the postmolt period cannot be stored, however, so that there is a period of high calcium demand. Carbon dioxide fixation in the carapace during the first few postmolt hours reduces the apparent respiratory quotient to from one-fourth to one-eighth of normal values, giving some indication of the magnitude of increased calcium requirement (*143*).

In the vertebrates, which are characterized by an endoskeleton, about 99% of the calcium, 80% of the phosphorus, and 70% of the magnesium in the body is bound in the hydroxyapatite crystallites of bone and teeth. For humans, the minimum requirements for calcium and phosphorus are stated to be 0.75 gm. each per day (they are actually about half of this), but this requirement is based primarily on maintenance of the blood and tissue levels, and even in growing animals only a small portion would be required for calcification. If a growing child consumed 1 gm. of calcium per day for a year, his annual consumption would be 365 gm., but only about 55 gm. or 15% of this would be retained, and his daily requirement for hydroxyapatite formation would be of the order of 150 mg. per day. The equilibrium between dissolved and precipitated calcium in vertebrates (and probably in many invertebrates) is maintained by a variety of factors (Ca:P ratio, vitamin D, parathormone), and disturbances in any one of these may produce "conditioned" calcification deficiencies. When tissue demands for calcium exceed the nutritional supply, calcium is mobilized from bone (but only slightly from dentine and enamel), and the serum calcium level may remain relatively normal in the presence of severe skeletal deficiencies. Beryllium salts induce rickets by interference with phosphate. Fluoride is not an absolute requirement for any species known, but nutritional supplies of about 1 p.p.m. in the water reduce the incidence of dental caries because of the greater insolubility of the enamel fluoroapatite which is produced on the surface by exchange of apatite ions. Other nutritional requirements for calcification in vertebrates have been studied at great length, and excellent reviews exist in the literature (*144*). The function of vitamin D in calcification, and the nutritional requirements for this substance, are discussed by Bauer *et al.* (*144a*).

VI. Requirements for the Catalysis of Organic Transformations

A. BASIC CONCEPTS

As early as 1869 von Liebig demonstrated that some unknown material present in animal extracts was essential for the proper growth and metabolism of yeast. Throughout the nineteenth century, however, there was little recognition that anything was required in the diet of animals other than carbohydrates, fats, proteins, salts, and water. Symptoms of scurvy and rickets have been observed in the skeletons of prehistoric skeletons, and the symptoms of these diseases, along with night blindness, were recognized by the medieval physicians, who prescribed goat liver for the latter condition. During the sixteenth to eighteenth centuries effective dietary measures were discovered to combat these disorders, but the rationale for therapy was generally based upon the neutralization of some noxious agent. Lunin demonstrated in 1881 that mice could not survive on a diet of carbohydrate, fat, protein, milk salts, and water, but that the addition of fresh milk kept the animals alive. From this he concluded that small quantities of unknown substances present in natural materials were essential to life, and Hopkins in more exacting experiments firmly established this basic concept some twenty-five years later. The earliest nutritional experiments on these trace substances employed a variety of vertebrates as laboratory animals (mice, rats, guinea pigs, pigeons, chickens). Comparative nutrition and the concept of the phylogenetic unity of biochemical needs did not arise, however, before the demonstration in 1919 by Roger Williams that the growth response of yeast to various natural extracts (containing thiamine) paralleled the activity of these preparations in experimental animals (*145*). This was further supported by the demonstration in 1933 that one of these factors (pantothenic acid, which was so-named because of the fact) occurred universally in material from all types of living organisms (*146*). The verity and value of this concept may be seen in the fact that microorganisms were instrumental in the discovery of biotin, folic acid, lipoic acid, pyridoxal, pyridoxamine, and pantothenic acid; that the protists formed the basis for the discovery of the vitamin properties of niacin, inositol and *p*-aminobenzoic acid; and that they facilitated the isolation of (and are the sole source of) vitamin B_{12}.

Early in the process of isolation of the "accessory growth factors" it became apparent that these substances were present in biological materials, and nutritionally active, in exceedingly small amounts. Isolation of thiamine required 3×10^4-fold concentration; biotin 1.4×10^6-fold. The obvious conclusion was that such substances acted catalytically. It has been pointed out that in the process of biopoiesis, the capture of solar energy was through photolysis, and its subsequent transfer and utilization were more or less directly between reactants to produce their products. The rates of reaction and reaction temperatures could range widely, and mineral surfaces provided loci for catalysis of a limited type. The evolution of metabolism in essentially isothermal systems necessitated the emergence of more specific and duplicable catalysts, and in the eobiont, minerals and their simpler organic complexes probably played this role. Apparently some common progenitor of the living forms of the present had evolved the enzymes and coenzyme systems that we now recognize, since "primitive" forms of these molecules are not recognized. The coenzymes, and the organic moieties that compose them, must, however, have evolved by a process akin to the "survival of the fittest," since for the most part their complexity is remarkably adapted to the reactions in which they serve. Even in the absence of the apoenzyme, for instance, thiamine or cocarboxylase will catalyze the formation of acetoin from pyruvate and acetaldehyde. Similarly, pyridoxal transaminates with amino acids in the absence of the apoenzyme, but the presence of a metal ion is required. Riboflavin per se may participate in oxidation-reduction reactions, and may do so in some tissues (e.g., the lens of the eye). The trace elements, however, may be the only true relic of the primordial biocatalysts.

The trace elements function largely as independent and dissociable parts of enzyme systems, or as more integral parts of prosthetic groups, as in the case of cobalt in vitamin B_{12} and iron in the cytochromes. For the most part they are elements that are required in the catalysis of reactions in which there is a considerable exchange of energy, and their function evolved in primordial systems because (*a*) they were present in sea water in measurable trace amounts (*147*), and (*b*) they exist in more than a single oxidation state and so may participate in electron transfer systems which are coupled with energy exchange. Elements with a single biologically available valence state tend to function in reactions in which there is no appreciable energy exchange; for example, chloride ion is required for amylase activity. In this regard, it must be emphasized that from the standpoint of comparative nutrition, many of the elements not generally categorized as "trace elements" must be considered. In the invertebrates and plants many "major mineral elements" are required only in trace amounts for catalytic functions. These same trace requirements exist in the vertebrates, but are most often ignored because of quantitative considerations. A few elements that manifest biological activity in trace amounts may participate in microstructures. The caries-preventive action of fluoride ion is due to the concentration of fluoroapatite in an exceedingly fine surface layer in dental enamel; silicon may have such a microstructural function in plants.

The nonprotein organic molecules which function in *general* catalysis are reasonably complex substances, and their biosynthesis requires a considerable number of sequential reactions. For this reason the probability of mutational loss of synthetic ability is high. Such a loss in a plant would be less common because of polyploidy. Lacking this factor; it would generally be fatal to that organism, since plants have a very limited ability to feed on other plants. Heterotrophic plants are thus uncommon. It is a basic characteristic of animals that they are phagotrophic, that is, that they ingest particulate food or "eat." Ingested organic molecules could thus circumvent a mutational block occurring in animals, although it makes them dependent upon other animals and plants. The evolution of more highly specialized animal species had as a corollary the need for additional biocatalysts, and these could be either synthesized by the organism itself (e.g., ascorbic acid), or modified from related materials which had uniquely evolved in the unrelated species that were ingested. In the first instance, the biological requirement having evolved at a late date, the total time for mutations to occur would be short, and only a few higher species would manifest a nutritional requirement, as is the case with ascorbic acid. In the second instance, when the practically preformed biocatalyst was obtained from another species, the nutritional requirement existed from the start and persists uniformly in all subsequently evolved species. Such a situation exists in the unique requirement of higher animals for vitamin A, which they derive from the uniquely plant carotenoids. Evolutionary adaptation of this structure to photoreception in animals would obviously have been an expedient process; the nonphotoreceptive function of vitamin A in animal tissues is still, however, completely unknown.

Early in the history of the elucidation of organic nutritional trace requirements it was proposed that these substances be termed "vitamin(e)s." At that time, the nature of their biochemical functions was of course unknown. The term "vitamin" was largely restricted to those substances that were required in vertebrate nutrition; expressions such as "accessory growth factor" were more commonly applied to compounds required only by more primitive forms. As the function of these latter compounds became clearer, and it became apparent that they were involved in vertebrate metabolism, if not nutrition, they too were more frequently termed "vitamins." Those materials that occurred in nature along with vitamin B_1 came by this association to be known as the "water-soluble" or "B complex" or just "B" vitamins, and eventually as constituents of all living things, even though they were not nutritionally required by many. A dichotomy thus arose between the B vitamins and the remainder, the former being involved in general metabolic catalysis, the latter in specialized systems. Subsequent developments have shown, however, that assignment of vitamins to one group or the other was not entirely justified on the basis of natural association with thiamine and of water solubility, and it seems probable that certain of the fat-soluble vitamins, such as the quinone vitamins E and K for instance, might more appropriately be assigned to the "general catalysis" group. As vitamin functions become better understood, further changes may be expected. A great many simple organic catalysts have undoubtedly not as yet been discovered, and it seems likely that some of these will be found to be nutritional requirements for one species or another, and thus, new vitamins. From a broad conceptual standpoint, then, a vitamin must be regarded as a lower molecular weight organic substance, involved in either general or special biocatalytic reactions, and not synthesized by the tissues of at least some species in which it has a functional requirement. The comparative biochemistry of the various chemical structures that compose the vitamins (carotenoids, flavins, pterins, sterols, etc.) is discussed in other chapters of this treatise.

The biochemical basis for the chemical needs for individual vitamins might appropriately be discussed on the basis of the types of organic transformations in which they are involved as catalysts (*148*). For intermolecular and intramolecular hydrations and dehydrations, no vitamin requirements have been reported, with the possible exception of inositol and vitamin B_{12}. Trace element requirements have been reported in instances of this type, however. Requirements are also unknown for intramolecular isomerization. Niacin, riboflavin, porphyrins, and quinones are required for simple oxidations and reductions involving electron transfer to and from carbon, oxygen, nitrogen, and sulfur double-bond pairs. α- and β-keto acid decarboxylations appear to require (in some cases at least) thiamine and biotin, respectively. Activation of the α-carbon of amino acids involves vitamin B_6 in decarboxylations, transaminations, and the tryptophanase reaction. Cleavages and condensations of the aldol type appear to require no vitamin, whereas those involving acetyl derivatives are associated with pantothenic and lipoic acids. Reactions involving single-carbon fragments often involve *p*-aminobenzoic acid, folic acid, or vitamin B_{12}. The biochemical function of the remaining vitamins is obscure.

B. The Trace Elements

1. *Sodium*

Sodium is so ubiquitous in nature that it is the most difficult of all trace elements to exclude from experimental diets. For this reason, the oft-repeated statement that it is not required by higher green plants and many other species still remains open to considerable doubt. As yet, it has been assigned no specific catalytic role in metabolism. Sodium is required in trace amounts by a number of the blue-green algae which have been studied, including *Anabaena cylindrica* (*56*), and the existence of this sodium requirement may be a phylogenetic characteristic of this class of organisms (*149*). About 5 p.p.m. of sodium are required in the medium for optimum growth, and the requirement is not replaceable by potassium, lithium, rubidium, or cesium. Sodium does not replace any part of the potassium requirement for this species. Sodium-deficient *Anabaena* contains less phycocyanin than do normal algae, but chlorophyll levels are unaffected. Other algae have been found to require sodium in order to sustain high photosynthetic rates, but not for the maintenance of respiration. In *Chlamydomonas* magnesium lowers and potassium elevates the sodium requirement (*150*). Sodium will replace part of the potassium requirement of *Aspergillus niger*, but it (and NH_4^+) increases the potassium requirement of lactobacilli. A trace sodium requirement has been demonstrated in a number of bacteria, including *Escherichia coli* (*58*). Among the insects, trace requirements have been observed in *Phaenicia cuprina* (blowfly), *Drosophila melanogaster* (*137*), *Theobaldia incidens,* and *Aedes concolor* (*151*); and favorable effects have been reported on the survival of other mosquito species. *Culex fatigans,* however, survives on media made up with distilled water and, like *Aedes aegypti,* requires only the slightest traces of sodium if any at all. *Blattella germanica* survives on levels of sodium below 5 μmoles per gram of diet (*152*). L strain and HeLa tissue cultures (mouse and human) have been shown to have a definite requirement (*153*).

2. *Potassium*

Potassium is required for the action of pyruvic kinase and possibly fructokinase. In plants it functions in both carbohydrate synthesis and translocation. There is thus ample reason to believe that its requirement, at least in traces, is universal. It is required by all higher plants, and in a plant deficiency the leaves rapidly turn a dark blue-green to bronze color, and exhibit marginal chlorosis and necrosis. Its requirement has been demonstrated also in algae (including *Chlorella*) and in all bacteria, yeasts, and fungi adequately investigated (e.g., *Bacillus cereus* for spore formation, enteric organisms; *Saccharomyces, Azotobacter* sp., etc., for growth). Some workers still question its universal requirement for the bacteria, however (see reference 5, p. 622). Rubidium and cesium can partially replace it in some species. The requirement is sufficiently specific in *Aspergillus niger* to permit the utilization of this species as a potassium assay organism (*154*). *Tetrahymena geleii* requires 0.3 μmole per milliliter of potassium for optimum growth, and other protozoa have similar requirements (*155*). The potassium needs of insects have not been satisfactorily investigated, but potassium is required by certain *Anopheles* species, by *Theobaldia incidens,* and by

Pseudosarcophaga affinis. It is closely balanced against calcium in the maintenance of cellular (protoplasmic) excitability or irritability in higher animals. Aside from this, its requisite nature in animals seems established on the basis of its function in pyruvic kinase which is essential to animal metabolism. Potassium is a demonstrated trace requirement for vertebrate tissue cultures (*153*).

3. *Calcium*

Calcium may be a universal trace requirement, although this is by no means certain. Its known biochemical functions are all associated with systems peculiar to the higher animals. It activates lipoprotein lipase (plasma "clearing factor"), as do magnesium or manganese, and it is essential to the action of bacterial collagenases. It stimulates the ATPase activity of both myosin and actomyosin, and is necessary for the enzymes involved in nerve impulse transmission. Normal neuromuscular irritability is dependent upon its presence in proper relationship to the monovalent cations. It is essential to blood clotting as well as to the clotting of milk. In this latter instance, barium will replace it. Finally, the production of milk by the mammary gland is dependent upon it. Calcium is believed to be an essential trace element in all higher plants and in most bacteria and fungi. When inadequate levels are available to green plants, the leaves become chlorotic, rolled, and curled; the roots are poorly developed and fruiting is inhibited. Reports that calcium is not required by algae and other lower plants are probably inaccurate owing to the tremendous experimental difficulties encountered in avoiding trace calcium contamination of media. *Anabaena cylindrica* requires about 20 p.p.m. in the medium, and this requirement is not replaceable by strontium. Calcium is essential for growth and spore formation in *Aspergillus* and *Penicillium* species, for nitrogen fixation by *Azotobacter*, *Rhizobium*, and others, for nitrification by *Nitrosomonas* sp., for naphthalene oxidation by *Pseudomonas* (*34*), and for the growth of cultures of many other species. Reports on the calcium requirements of Protozoa are conflicting (*155*), but the consensus is that trace requirements do exist. Calcium has been demonstrated to be a chemical need for seven species of anopheles mosquitoes, *Aedes aegypti*, and *Theobaldia incidens*, but not convincingly for *Drosophila*. For *Blattella germanica* about 50 p.p.m. or less are required in the diet. Calcium is required in all tissue culture media for sustained indefinite growth (*153*).

4. *Magnesium*

Magnesium is required as a structural portion of chlorophyll and in combination with nucleic acids in various cytological structures. It is necessary for the action of a number of hydrolases (e.g., aminopeptidases, lipoprotein lipases, and bacterial gelatinases), presumably forming an enzyme-magnesium-substrate intermediate in which the positive charge of the carbonyl carbon is enhanced. Magnesium functions in most, if not all, reactions involving high-energy phosphate bonds because it is a cofactor for oxidative phosphorylation (coupling factor) and thus appears to be one of the only mineral elements for which there is an unequivocal universal nutritional requirement. In some instances, manganese or other ions may substitute for it. Deficiency symptoms in green plants are much the same as for calcium, manganese, or iron, and the requirement in this case is relatively high because of the need for chlorophyll synthesis. Magnesium and calcium are also components of the pectate salts in the structure of the middle lamella. In the plants, as in all cellular materials, the magnesium : calcium ratio is critical (*149*). In bacteria, magnesium is required for nitrogen fixation and for pigment formation, and the element may be assayed using *Aspergillus niger*. *Tetrahymena geleii* requires about 2 μg. per milliliter of medium for normal growth. The requirement is well established in several insects, although amounts may be so low as to be difficult to detect, as in *Aedes aegypti* (*156*). Traces are also known to be required in mammalian tissue cultures.

5. *Halogens*

The halogens have in no case been shown to be universal nutritional requirements. Each one, however, has a beneficial effect in at least some species.

a. Fluorine. Fluoride is beneficial to apatitodentulous animals in concentrations of about 1 p.p.m. in the drinking water, because of the formation of an acid- and caries-resistant outer layer of enamel fluoroapatite. Fluoridation of low-fluoride public water supplies is a common practice for this reason. Fluoride is present in sea water in about this same optimal concentration (*147*). Levels appreciably above this, however, produce fluorosis in the enamel surface. Evidence has been presented that fluoride is nonessential in the nutrition of rats (*157*). Diets containing less than 0.007 p.p.m. of fluoride maintained rats through three generations and the beginning of the fourth with no sign of differences from fluoride-diet controls. A similar situation may not hold in all organisms, and the recent demonstration of naturally occurring fluoro fatty acids indicates a need for further investigation (*158*). These substances are present in certain South African plants, are poisonous to cattle that graze upon these plants, and produce a paralytic state in man known as "brokeback." The high toxicity of fluoride ion (it ranks among the elements even higher than copper) is generally attributed to its formation of magnesium fluorophosphate in critical glycolytic enzyme systems such as enolase.

b. Chlorine. Chloride is required in trace amounts for the activation of certain amylases, but its universal requirement has never been demonstrated. It is stated to be nonessential for all plants, although contrary reports are available for *Escherichia coli* (*58*) and the halophilic bacteria (*60*). Chlorocarbon compounds occur naturally (e.g., chloramphenicol in *Streptomyces venezuelae* and chlortetracycline (Aureomycin) in *Streptomyces aureofaciens*), but biological functions for these are unknown. Chloride requirements are assumed to exist in most animals because of the participation of that anion in ion equilibria, and the frequently high requirement for gastric acid, but the chloride need has seldom been assessed separately from the need for other ions. In the German cockroach it has been shown that the elimination of chloride from the diet is more damaging to growth and survival than elimination of both sodium and chloride (*152*), and that the chloride requirement is probably less than 5 μmoles per gram of diet. Chloride has not been shown to be dispensable in any tissue culture study.

c. Bromine. Bromide may substitute for chloride in some of its functions (e.g., in amylase activity), but it is not believed to do so in nature. It is present in sea water to the extent of 50–100 p.p.m. and is concentrated by many marine algae. It is present as dibromotyrosine in the protein gorgonin of certain corals and as the dibromoindigo pigment, punicin, obtained from various gastropod mollusks. The essentiality of bromine for these species seems reasonably certain. In higher animals, despite sporadic claims to the contrary, bromine is not known to be required, and rats have been raised through an entire generation without any deficiency symptoms on a diet containing less than 0.5 p.p.m. The young of such rats contained less of it than the young of a similar group fed the diet plus 20 p.p.m. of bromide (*159*).

d. Iodine. Iodine is not known to be required by plants although the normal growth of maize has been reported to require it. It is present in sea water to the extent of about 0.05 p.p.m. and is concentrated from this by marine plants (*160*) to concentrations of up to 0.2%. It is also concentrated by other plants to a lesser degree depending upon concentrations in the environment, and by marine and other animals, particularly in the liver oils (*161*). Traces of iodine (0.001 to 1 p.p.m. in the medium) are said to stimulate yeast and bacterial growth (*5*). Iodine in the form of diiodotyrosine is found in the iodonin or gorgonin of certain corals, and is known to be required by higher animals for the synthesis of the various thyroid-stimulating iodoamino acids and proteins. Iodoproteins and iodoamino acids promote the growth and development of *Corcyra cephalonica* (the rice moth), but are not essential (*162*). Vertebrate requirements are about 20–40 μg. of iodine per 1000 food calories consumed; they are also reported as about 1.0 μg. per day for the rat, and 3.0 μg. per kilogram per day for humans. Nutritional deficiency results in hypothyroidism (cretinism, myxedema, goiter), and is a frequent deficiency disease in humans. Iodized salt has provided an expedient and efficient solution to the problem of endemic goiter. Hypothyroidism has been induced by dietary means in rats (*163*) and in hamsters (*164*), and brought about in other animals by the employment of synthetic goitrogens and by TSH. A naturally occurring goitrogen (L-5-vinyl-2-thiooxazolidone) has been isolated from the roots and seed of turnip and from the seed of other members of the Brassicae (*165*).

6. *Zinc*

Zinc is a universal trace requirement. Seven zinc metalloenzymes have been studied in detail, including carbonic anhydrase, carboxypeptidase, alcohol dehydrogenase (yeast and liver), glutamic dehydrogenase, lactic dehydrogenase, and alkaline phosphatase (porcine kidney). Numerous other enzymes are activated by zinc, but also by other metals. Zinc is said to be a constituent of the respiratory blood pigment (hemosycotypin) of certain mollusks and to occur in cobra venom (0.5%). There is twenty-five times more zinc in human leukocytes than in erythrocytes, owing to the presence in the former of a protein containing 0.3% zinc. This element plays a definite but unclear role in pancreatic and insulin physiology. The tissues of the eye are high in zinc (cf. V,C), but there are marked differences in these tissues and between species.

In green plants, zinc deficiency is common and is compounded by the zinc requirement for tryptophan synthetase activity and thus for indoleacetic acid (heteroauxin) synthesis. Typical major outbreaks of deficiency disease were the mottle leaf disease of citrus trees (California) and the rosette leaf disease of pecans (Texas), although syndromes are known for many other plants. Zinc is required by algae, bacteria, and fungi. *Aspergillus niger* has been used as an assay organism, and its need for zinc was discovered (1869) sixty-five years prior to the demonstration of a need in higher animals (1934). Zinc is required by all insects investigated in this regard, including *Alabama argillacea*, *Blattella germanica*, *Bombyx mori*, and *Corcyra cephalonica*. The manifestation of carnitine deficiency in Tenebrionidae depends on zinc (and potassium) in the diet, the optimum zinc level being about 5 p.p.m. Nutritional deficiencies have been experimentally produced in the mouse and rat, which require 4–6 p.p.m. in their diets. For the rat, 20 μg. of zinc per day permits a severe deficiency which is improved by elevation to 30 μg. and completely prevented by 40 μg. Levels of 80–100 p.p.m. of zinc are required to prevent deficiencies in pigs. Porcine parakeratosis appears to involve a direct or conditioned zinc deficiency aggravated by the high calcium levels in the diet due to added bone meal. It is alleviated by supplementation of normal diets with 0.02% of zinc carbonate. Deficiencies occur in chicks on soybean and sesame meal diets, and the effect of calcium is manifest only in the presence of plant proteins, which presumably bind the zinc. Zinc deficiencies in humans have been associated with subacute and chronic beriberi, nutritional edema, semistarvation, and (most commonly) postalcoholic cirrhosis. The latter condition may create a conditioned zinc deficiency, since blood and liver levels are below normal, and zinc excretion is greatly elevated; and these values vary with the course of the disease. The biological role of zinc has been extensively reviewed in the literature (*166*).

7. *Iron*

Iron is present in the cytochromes and in cytochrome oxidase, and on that basis alone it is nearly a universal requirement. It is a part of several enzyme systems, including catalase, aconitase, peroxidases, and numerous flavoprotein dehydrogenases. It is a constituent of ferritin, myoglobin, the hemoglobins of many vertebrates and invertebrates, and the erythrocruorins, chlorocruorins, hemerythrins, and echinochrome of various invertebrate groups. Iron is required by green plants for chlorophyll synthesis, deficiencies producing chlorosis. It is needed by algae (*167*), fungi, and yeasts. It is essential to nitrogen fixation by *Azotobacter*, pigment (prodigiosin) production by *Serratia marcescens* and other organisms, and toxin formation by *Corynebacterium diphtheriae*. The optimal concentration in this last instance is $2 \times 10^{-6}\,M$. *Escherichia coli*, *Aerobacter aerogenes*, and *Klebsiella pneumoniae* require in their media optimum iron concentrations of 0.02–0.03 p.p.m.; *Pseudomonas aeruginosa* needs 0.1 p.p.m. It is conceivable, however, that some anaerobes may not require iron.

Tetrahymena geleii requires iron, but protozoan needs have not been adequately evaluated. Among the insects, the mosquitoes *Aedes concolor* and *Culex fatigans* have requirements too low to be accurately measured. The rat flea *Nosopsyllus fasciatus* requires blood only as an iron source, readily utilizing ferrous ammonium sulfate as a dietary substitute. [The blood-sucking bug *Triatoma infestans* and many bacteria and protozoa require iron in the form of heme (cf. VI,C,3), and cannot substitute inorganic iron salts.] Iron deficiencies in man are manifest primarily by anemia. The physiological iron requirement of the adult male is 0.9 mg. per day; for females 1.5 mg. are required to meet the added need due to menstrual blood loss. During gestation, 60% more iron is required than would have been lost during menstruation over the same period. Various factors in iron metabolism cause the nutritional requirement to be several times greater than the physiological requirement for iron, and this latter is frequently near the iron content of the average diet. In many parts of the world, fortification of white flours with iron largely eliminates this problem.

8. *Copper*

Copper is a constituent of various oxidases that are widely distributed in nature, including uricase, phenol oxidases, and ascorbic acid oxidase. Butyryl CoA dehydrogenase normally contains copper in the molecule, but the enzyme is equally active without it. Cytochrome oxidase contains both bound copper and heme (*168*). Tyrosine-α-ketoglutarate transaminase is also said to contain copper. A cuproporphyrin feather pigment, turacin, is found in touraco feathers and contains 7.0% copper (*169*). Copper is a component of the blue, nonporphyrin, respiratory pigment hemocyanin, of certain gastropods and arthropods; and copper is found in high concentrations in the hepatopancreas and gonads of some mollusks and in the liver of higher animals. It is present in mammalian blood serum in the form of the α-globulin, ceruloplasmin, and the β-globulin, transferrin, and in the erythrocytes as erythrocuprein.

Copper is required by higher green plants for several purposes, and soil deficiencies cause wilting of terminal shoots, blanching, and impaired carotene and pigment formation. It is required by all algae studied in this regard, but the ranges between needs and toxicities may be quite narrow and highly species specific. Thus, concentrations that stimulate *Spirulina* and *Oscillatoria* sp. are inhibitory to *Phormidium tenue* (*170*), and this may be an important ecological factor in the sea (*149*), where concentrations vary seasonally as much as tenfold. Copper is required by yeasts, molds, and bacteria for growth and metabolism and, in special cases, for nitrification and aromatic hydrocarbon oxidation. It is critical for differentiation and pigmentation in *Aspergillus niger* (*171*), and this organism has been employed as an assay organism (*154*), giving responses in the range 1–50 μμg.

As in the protistan plants, copper is remarkably toxic to the invertebrates. It is required in minute amounts by the Protozoa, but these are so small that, as Kidder remarks (*155*), "one is fortunate to be able to prove such a requirement and can only do so with media containing amino acids of certain brands." It is required by hydra, but in this case even ordinary distilled water may contain enough copper to be toxic (*172*). Its need is particularly obvious for the hemocyanin-containing arthropods and mollusks. Copper needs have been indicated in *Blattella, Drosophila,* and *Corcyra.* In vertebrates, copper deficiencies are associated with anemia, achromotrichia, alopecia, and demyelinating diseases. Deficiency in grazing animals is quite common; it is often complicated by a need for other trace elements (e.g., cobalt) and caused by: (*a*) outright soil deficiency (e.g., enzootic ataxia of lambs in Australia); (*b*) combined deficiency (e.g., with cobalt in "saltsick" of Florida cattle); and (*c*) conditioned deficiencies due to excessive amounts of other elements (e.g., molybdenum antagonism of normal copper pasturage, producing "swayback" in lambs). Copper is required for the incorporation of iron into hemoglobin, and milk diets have been shown to precipitate, for this reason, a microcytic anemia. In Wilson's disease, there is a hereditary disorder of copper metabolism, associated with low ceruloplasmin levels and increased liver and brain storage. Although copper is doubtless required by mammalian tissue cultures, the levels are very low and even minute amounts are toxic. The high toxicity of copper and of several related metals is manifest in nutritional studies with chemically defined diets because of the lack of the metal-binding effect of protein and other large molecules absent from these diets. Antagonisms between trace amounts of metals may become more pronounced under these circumstances. In contrast to most mineral elements, copper is less concentrated in milk than in blood, thus tending to favor deficiencies in the young. Requirements for sheep and cattle are about 6–8 p.p.m. in the pasturage (dry basis), with needs being increased somewhat for wool sheep (in which deficiencies are economically disastrous). Acute copper deficiency in humans has not been observed, but requirements must be lower on a weight basis than for grazing animals. This is contrary to what would be expected, since reasoning by analogy with other catalysts (e.g., vitamin requirements) would suggest a reciprocal relationship between species size and nutritional requirement on a weight basis. The specialized needs of individual species may create anomalies in such a trend, however, and pelt production may be involved in this instance. Human daily requirements are generally stated to be 0.05 mg. per kilogram body weight for infants and 1–2 mg. per day for adults. Quantitative needs have been assessed for a variety of smaller laboratory and domestic animals.

9. *Manganese*

Manganese catalyzes the decarboxylative step performed by the oxidative β-decarboxylases (malic, isocitric, and phosphogluconic dehydrogenases) by forming, with the intermediate β-keto acid, a chelation complex which spontaneously decomposes to eliminate CO_2. It activates glycylglycine dipeptidase, leucine aminopeptidase, arginase, enolase, lipoprotein lipase, deoxyribonuclease, ureidosuccinase, serine hydroxymethylase, and many other enzymes. It is involved in fatty acid synthesis from acetyl CoA, and sphingosine synthesis from palmityl aldehyde and serine. Manganese does not appear to be concentrated in specific tissues although higher levels occur in liver in association with arginase. Every animal (but not plant) tissue possesses a concentration of manganese characteristic for that organ and similar to the levels found in that same organ in other animal species, suggesting indigenous tissue control of that concentration as a tissue characteristic. Manganese metabolism is in some unknown manner closely bound to the metabolism of choline and inositol. Chelation of manganese with many different molecular structures may cause the ion to give misleading effects, such as are seen in the Aureomycin-induced protection against perosis in chicks.

Manganese is required by higher plants, deficiencies resulting in mottled chlorosis and reduced carotene formation. It is present in particularly high levels in tea leaves (80–140 p.p.m. dry weight) and coffee beans (14–32 p.p.m.). It is also required by algae (*167*), bacteria, yeasts, and molds. A strain of *Lactobacillus arabinosus* has been employed both for freeing manganese from a sample and for final assay, being suited for values in the range of 0.05–0.5 μg. (*173*). Manganese is required by *Blattella germanica* and is concentrated by wasps at certain stages during their development (*174*).

In vertebrates, manganese is particularly necessary for normal reproduction, lactation, bone formation, nervous coordination, and growth. Deficiencies are strikingly manifest in these processes and have been studied in mice, rats, rabbits, swine, cattle, chickens, and ducks. Deficiencies are unrecognized in man, but this fact requires further investigation. The human requirement is estimated to be about 4 mg. per day. In all mammals, requirements have not been adequately assessed because of their tremendous dependence on the levels of calcium and other substances in the diet. The only naturally occurring deficiency that has been reported in mammals was in cattle grazed on plants that were grown on sand and peat soils low in available manganese (*175*).

Diets used in domestic bird and egg production may be inadequate to meet the unusually high requirements of these species, resulting in such abnormalities in bone growth as: (*a*) *perosis*—enlarged and malformed tibiometatarsal joints with slipping of the Achilles tendon from its condyles; and (*b*) *chondrodystrophy*—increased percentage of de-formed embryos, with shortened legs and wings, and globular heads. Other symptoms include ataxia, lowered egg production, decreased hatchability, and reduced eggshell breaking strength. Dietary manganese requirements are high for birds (about ten times those of mammals), partly owing to the fact that only 20–25% of that ingested is utilized. There is also considerable strain variation and an increased requirement for egg production. Injection of 30 μg. of manganese ions into a deficient egg will permit normal development. Chicks require 30–50 p.p.m. in the diet, depending upon the strain; pullets in the first year of egg production need somewhat more; and hens in the second year require about 70 p.p.m. The biology and toxicology of manganese have been extensively reviewed in the recent literature (*176*).

10. *Molybdenum*

Molybdenum is known to function in nature only as a part of a number of molybdoflavoprotein enzymes, occurring in the oxidized flavoprotein as the hexavalent ion. In this capacity, it is in association with flavin adenine dinucleotide (FAD) and sometimes iron. Notable among these enzymes are: (*a*) xanthine oxidase, which oxidizes many different purines and aldehydes; (*b*) an aldehyde (but not purine) oxidase; (*c*) nitrate reductase (plant and *Neurospora*) and nitrite oxidase (*Nitrobacter*); and (*d*) hydrogenase (*Clostridium*). Molybdenum may often "malfunction" in nature through ion antagonisms. Higher green plants require molybdenum in measurable trace amounts for nitrogen metabolism, and the deficiencies that have been observed are characterized by a light yellow chlorosis and often by a failure of the leaf blades to expand. Molybdenum requirements have been assessed for many algae, including *Anabaena*, *Chlorella*, *Nostoc*, and *Scenedesmus*. In *Anabaena cylindrica,* the requirement is not replaceable by vanadium, as it is in most other organisms. For this organism, the requirement is about 0.2 p.p.m. in the medium when grown on elemental nitrogen, and only half this when grown on nitrate; in the presence of ammonia it is much less, if indeed it exists at all. In algae, the effect of molybdenum may in part be indirect, exerted through inhibition of phosphatase activity (*177*). Molybdenum is required by certain nitrogen-fixing (*Azotobacter*, *Clostridium*, *Rhizobium*) and nitrite-oxidizing (*Nitrobacter*) bacteria and by certain fungi (*Aspergillus niger*), the need being partially replaceable by vanadium in somewhat higher amounts (*178*). In *Azotobacter*, 1–4 p.p.m. in the diet stimulates nitrogen fixation ten- to thirtyfold. Iron stimulates these organisms with either molybdenum or vanadium (but not both, which are inhibitory), owing to the involvement of the metalloenzyme-cytochrome sequence. Molybdenum deficiency may be induced in higher animals, such as the rat, by the inclusion of tungstate in the diet. The xanthine oxidase levels of tissues are appreciably reduced in such cases. A more important reciprocal relationship exists between molybdenum and copper. When pastural molybdenum levels are low (0.1 p.p.m. or less) and copper is normal or high (15 p.p.m. or more), a high copper status develops in grazing animals which may lead to copper poisoning in sheep or cattle (*175*). By contrast, molybdenum in normal or high levels, in the presence of dietary sulfate, tends to limit copper assimilation and liver storage and thus to create a copper deficiency with accompanying anemia and bone and muscle lesions. "Teart" in cattle represents such a condition, and is curable with massive quantities of copper sulfate (100–300 mg. intravenously; 1–2 gm. daily in the diet). Copper also reduces molybdenum toxicity in the rat.

11. *Cobalt*

Cobalt appears to function solely as a portion of several vitamin B_{12}-containing coenzymes which are apparently involved in amino and nucleic acid metabolism, oxidation-reduction of dithio groups, and the transfer of single carbon fragments derived by the rupture of carbon-carbon bonds. While the requirement for cobalt is probably universal, the absolute amount is so small that in many cases it is still not demonstrable. Conversion of cobalt to various forms of vitamin B_{12} is limited to the lower plants (microorganisms). Cobalt ions function, along with other metallic ions, in activating a number of enzyme systems (e.g., yeast aldolase, glycylglycine dipeptidase), but these functions are nonspecific. Many algae can utilize cobalt, but it can be replaced by much smaller amounts of vitamin B_{12}. Other algae are used as vitamin B_{12} assay organisms and thus cannot utilize the elemental mineral. Cobalt is required by bacteria and fungi for vitamin B_{12} synthesis, and the effects of cobalt in higher animals are due solely to microbial synthesis by symbiotic microorganisms. (A few bacteria, however, cannot effect the synthesis.) It is only in this sense that cobalt may be said to be required by animals. Natural deficiencies of cobalt are seen in cattle, goats, and sheep grazing on pasturage containing less than 0.07 p.p.m. of cobalt (dry basis), and also in conjunction with other trace element deficiencies, but horses and pigs do not show deficiency symptoms on such diets and rats and rabbits have a requirement far below this. Man apparently requires less than 0.2 μg. per day (as B_{12}), which is about one five-hundredth the amount required by sheep. Relatively high amounts of cobalt stimulate hematopoiesis and produce polycythemia, but not in ruminants.

12. *Selenium*

Selenium is active in trace amounts in preventing and curing some of the symptoms of vitamin E deficiency: hepatic necrosis in rats and swine, exudative diathesis in chicks and turkeys, and muscle disorders in rabbits and ewes (*179*). It does not protect vitamin E-deficient rats against fetal resorption, give complete protection in rabbits, or protect E-deficient chicks against encephalopathy. Selenium-deficient diets, in which the hay contained less than 0.1 p.p.m. of selenium, have produced "white muscle disease" in lambs, and the condition has responded to selenium (*180*). It has been suggested that selenium may function as a portion of the succinoxidase electron-transfer system, but, by contrast, that it may only be a nonspecific antioxidant "like vitamin E." Quantitative considerations do not favor the second alternative. An as yet impure, water-soluble, organic compound, α-factor 3, which has been extracted from acid hydrolyzates of hog kidneys, contains 0.2% selenium. It is active in the prevention and treatment of the previously mentioned symptoms at levels in the diet corresponding to 0.007 p.p.m. of selenium. Other selenium compounds, including selenate, are only about one-third as active.

Selenium has been reported to favor the germination of spores and the growth of some molds (*5*). It partially replaces sulfur in the cysteine and methionine of plants grown on seleniferous soils, and in animals. It is highly toxic to all animals in low concentrations, and this toxicity is completely prevented by the inclusion of 5 p.p.m. of arsenic in the drinking water. These levels are nontoxic, and provide a simple solution to a major health problem (selenism) in certain parts of the world.

13. *Vanadium*

Vanadium is required by higher plants and by algae. It is also utilized by *Azotobacter* for nitrogen fixation (in conjunction with molybdenum) and by *Aspergillus niger*. Requirements that have been demonstrated are exceedingly low (10^{-5} p.p.m. in the medium for *Azotobacter*), and it is possible that vanadium requirements may be quite general. It is concentrated in certain ascidian orders (Phlebobranchiata and Aplausobranchiata) in the vanadocytes in conjunction with high concentrations of sulfuric acid and other related metals (titanium, chromium, manganese, iron, niobium) (*181*). Its function in the tunicates is uncertain, but its presence is not considered to be adventitious.

Vanadium-porphyrin complexes occur in certain ancient oil shales in large amounts (*34*).

14. *Boron*

Boron in trace amounts has long been known to be essential for all forms of higher plants, but its exact function is unknown. Algae suffer impaired growth and become chlorotic in its absence. Repeated attempts to demonstrate its requirement by animals have failed, although beneficial effects have been noted in potassium-deficient animals (*182*).

15. *Silicon*

Silicon is apparently essential for the higher plants (cf. V,D) and for the reproduction of certain marine protists. It constitutes a major portion of the skeletal structure of some of the latter and of some sponges and occurs in lesser amounts in all higher animals.

16. *Gallium*

Gallium is said to be required by *Aspergillus niger* (*183*).

17. *Miscellaneous*

Most of the other elements have been claimed to have trace element functions at one time or another, but the essentiality of these has never been proved. Claims for nickel, arsenic, and aluminum have been most prominent in animals, but far from conclusive. Many of these reports are based upon the stimulation of cellular growth (arsenic, thallium, titanium, radium) (*5*). To some extent the observed effects may be due to the relatively nonspecific trace-element activation of certain enzyme systems. In other instances, changes in the balance of ion antagonisms may be responsible (Na–K, K–Rb, Ca–Mg, Mo–Cu, Zu–Cu, Cu–Fe, Cu–Zn, Zn–Ca, V–Mo, etc.) (*184*). These relationships may be misleading; thus, rubidium is inactive for some organisms, antagonistic to potassium in others, and synergistic with potassium in still others. Further, beryllium and cadmium are reported to replace zinc for some molds; chromium stimulates respiration in *Rhizobium;* cesium stimulates some yeasts and algae, but—with lithium, barium, and strontium—antagonizes sodium and potassium in *Spirogyra.* Similar relationships are manifold.

Many elements are accumulated by certain species in amounts greatly above those which occur in the environment. Common examples are the accumulation of vanadium by the basidomycete *Amanita muscaria*, of selenium by ragweed, and of aluminum and the rare earths by the hickory. Sometimes the accumulation mechanism is apparent and the process is adventitious, as in the accumulation of fluoride, lead, radium, and strontium in bone. In other cases the process is unclear, and it is worthy of examination with the hopes of discovering some indication of biological function. Impetus to these studies has been given by the appearance in the biosphere of radioactive isotopes which may be harmful in accumulated amounts because of radiation damage (e.g., strontium-90). Many organisms have recently been discovered to be accumulators of very rare elements, and fractionation of the isotopes of a single element is sometimes observed. It seems apparent that other trace element requirements will eventually be found as techniques are developed that would permit of their discovery.

C. Vitamins Involved in Electron Transfer Systems

1. *Nicotinic Acid Amide (Niacinamide)*

This vitamin is known to function only as part of the adenyldinucleotides, DPN and TPN, in electron transfer between a reduced and an oxidized metabolite, or between a reduced metabolite and an FAD enzyme. Some dehydrogenases may utilize either of the coenzyme forms, others function better with one than the other, and a few function only with one. The reasons for this DPN:TPN distribution are unclear, but it has been pointed out that the TPN-TPNH system may be associated with a "high pressure line" functionally connected to biosynthetic pathways, as opposed to oxidative energy generation (*185*). In living animal tissues, all the niacinamide is in the form of the nucleotides, but after cellular death these are rapidly decomposed to liberate free niacinamide. Nicotinic acid is absent. In plants, in addition to the amide form and its nucleotides, there also occur free nicotinic acid and other substances which yield nicotinic acid on hydrolysis. Nicotinic acid can be converted to the amide *only* by preliminary conversion to nicotinic acid mononucleotide and then DPN, followed by hydrolytic cleavage.

In vertebrates and fungi, niacin is synthesized by the degradation of tryptophan to kynurenine and subsequent rearrangement of the latter to form quinolinic acid (*186*). This biogenetic sequence has apparently been lost in the cat (*120*). The conversion does not occur in most insects (*Blattella, Drosophila, Phormia, Tenebrio, Tribolium*), although a number of the metabolic intermediates (including 3-hydroxyanthranilic acid) are formed as precursors of eye pigments or as cuticular tanning reagents (*114*). The mechanism is uncertain in the silkworm, but in the rice moth (*Corcyra cephalonica*) conversion does occur, and the metabolic details of the process have been extensively investigated (*187*). The conversion of tryptophan to niacin apparently does not occur in the lower invertebrates (the nematode *Caenorhabditis briggsae* (*188*), *Tetrahymena*) or in mammalian cell cultures. It does occur among the fungi (*Neurospora, Phycomyces, Trichophyton*). In most bacteria and higher plants, but not in animals, niacin is synthesized by a very different mechanism involving condensation of a three-carbon and a four-carbon fragment (*189*), but probably not from ornithine as it appeared earlier (*186*).

The higher plants have no nutritional need for niacin, although it may be required by plant embryos, roots, cuttings, and pollen grains (*190*). Many bacteria, yeasts, and fungi do not require a nutritional source of niacin. Members of the *Hemophilus* group, *Borrelia*, and other highly parasitic organisms may require DPN or TPN ("Factor V"); they can use the mononucleotide to a limited extent, but cannot synthesize it from niacin or niacinamide. Some strains of *Pasteurella* can utilize niacinamide, but not nicotinic acid; and strains of *Leuconosotoc mesenteroides* can use niacin, but not the amide. Niacin, its amides and nucleotides may have varying activities for some organisms, but for most bacteria and for animal species they are about equally active. Some bacteria and fungi may utilize niacin derivatives that are inactive for animal species (e.g., trigonelline), and so are unsuitable as microbiological assay organisms. *Lactobacillus arabinosus* seems to have the same niacin-utilizing capacities as higher animals. Thus, among the microorganisms, all degrees of nutritional dependence for coenzyme synthesis may be encountered. In the animals, niacin requirements are extremely dependent upon the extent of niacin synthesis from tryptophan and upon the supply of niacin provided by symbiotic microorganisms. The efficiency of the tryptophan conversion varies from about 1 to 5% of the tryptophan available, making adequate total synthesis difficult in some less-efficient species (*191*). As might be anticipated, the requirements on a weight basis are larger for smaller animals (*192*), and blood and tissue concentrations follow this trend (*193*). Absolute requirements for humans have been variously estimated at from 1.5 to 20 mg. per day. Requirements for bacteria and for *Tetrahymena* are about 0.1 μg. per milliliter of medium. Although characteristic nutritional deficiencies have been induced in many insects, fishes, birds, and mammals, they are known to occur naturally only in man (pellagra) and the dog (blacktongue). Absence of adequate nutritional supplies of *both* niacin and tryptophan are generally essential for the development of frank deficiencies. Deficiency symptoms have been described for several dozen species (*186*).

2. *Riboflavin (Vitamin B_2)*

Riboflavin functions primarily as the flavin mononucleotide (FMN) and dinucleotide (FAD) in electron transfer between metabolites or reduced pyridine nucleotides and oxygen, cytochrome, or other "electron-transferring" flavoproteins. Riboflavin forms complexes with tryptophan, taking up one electron from it and forming a semiquinoid free radical, and such complexes may be significant in electron-transfer systems. In this regard, riboflavin may have special oxidation-reduction functions in the transparent portion of the eye along with tryptophan (cf. V,A,16). Riboflavin is involved in phototropism and indoleacetic acid metabolism in plants (*194*). It has been identified as a scale pigment in some reptiles, and in crystalline form as the reflecting substance in the tapetum of the lemur *Galago crassicaudatus* (*130*). The synthesis of riboflavin is an exceedingly complex process; it can be conveniently studied in the yeast *Eremothecium ashbyii* and the mold *Ashbya gossypii* (*189*). These organisms are used as industrial sources of the vitamin, which they produce in crystalline form in their culture media. Although higher plants can synthesize riboflavin, some nonphotosynthetic portions of plants cannot, and the synthesis in entire plants is often insufficient to permit of optimum growth rates. Riboflavin is required by some bacteria, yeasts, molds, and algae. It is a universal nutritional requirement for the animal kingdom (protozoans, nematodes, insects, vertebrates). Synthesis of the coenzyme form can apparently be achieved by all species. Synthesis by intracellular and intestinal symbionts may supply a major portion of the need. The absolute requirement for humans is about 1 mg. per day, and the requirement on a weight basis varies inversely with species size (*192*). Human ariboflavinosis most often occurs in association with other nutritional deficits (e.g., famine, beriberi, pellagra, perleche).

3. *Porphyrins*

Porphyrins provide the organic framework for the incorporation of iron into the cytochromes, which are involved in electron transfer between flavoproteins and oxygen. The cytochromes are thus components of all known aerobic organisms. Porphyrins also provide the structural basis for chlorophyll, for the oxygen-transporting respiratory pigments (e.g., chlorocruorin, and animal and legume hemoglobins), the prosthetic groups of catalase and peroxidase, and vitamin B_{12}. The functions of all these substances involve electron transfer in some fashion. A copper porphyrin, turacin, also occurs as a blue feather pigment. With the exception of vitamin B_{12}, which has six extra, methionine-derived, methyl groups, the structures of all of the known naturally occurring porphyrins and their derivatives are isomerically related and are believed to originate from a common precursor (cf. V,A,1). Most plant and animal species can perform the porphyrin synthesis, but one insect and a few parasitic bacteria and protozoa cannot. The bacteria include *Bartonella bacilliformis, Hemophilus canis, H. ducreyi, H. pertussis,* and *Pasteurella pestis.* Among the protozoa are *Leishmania donovani, L. tropica, Crithidia fasciculata,* and *Trypanosoma cruzi.* In fact, no leishmania or trypanosome is known that does not require exogenous hematin (*195*). The insect is the blood-sucking *Triatoma infestans,* a vector in the Chagas disease caused by *Trypanosoma cruzi.* Hematin ("Factor X") functions as an essential dietary precursor of porphyrin in all of these species and in very low concentrations (0.001 μg. per milliliter of medium). When *Hemophilus* is grown anaerobically, the requirement remains, but it is much lower owing to the lost necessity for cytochrome synthesis. Protoporphyrin IX is also active; these species are thus able to insert the iron into the protoporphyrin. The heme in either case has been demonstrated to be the cytochrome precursor, and it is also active in the synthesis of the other less-critical porphyrin compounds. Those protoporphyrins that do not possess a vinyl group usually do not support growth, although they are active if supplied with the iron already incorporated into the molecule. This suggests a function for the vinyl group, but it has been pointed out that nonvinyl porphyrins are not active in all cases. Cultures grown in their presence, for instance, cannot reduce nitrates.

Coprogen is an iron-containing compound that is widely distributed in nature and essential for the growth of certain coprophyllic fungi (*Pilobolus*) (*196*). Desferricoprogen is inactive, and 800-fold concentrations of inorganic iron are active, suggesting the involvement of this growth factor in iron transport.

4. *Quinones*

In the form of isoprenoid homologs of 2,3-dimethoxy-5-methylbenzoquinone (ubiquinones), quinones are widely distributed in nature and appear to be a functional portion of the macromolecular lipoprotein complex associated with the electron transport chain. They are believed to function between succinoxidase flavoprotein and cytochrome c_1 and perhaps in other sites. Mammalian ubiquinone contains ten isoprene units (coenzyme Q_{10}), *Torula* contains nine units (Q_9), *Azotobacter vinelandii* has eight (Q_8), and *Saccharomyces cerevisiae* has six. Although ubiquinone deficiencies have not been observed in any species, the substances are in one sense vitamins, in that the synthesis of the benzene ring cannot be effected by animals (*197*). The source of the ring structure is unknown (it is not from tocopherol), but the isoprenoid side chain is derived from mevalonic acid. Similar isoprenoid side chains are found in vitamin A (four isoprene units), tocopherols (four units), vitamin K_1 (four units) and vitamin K_2 (six units); and vitamins E and K have been suggested as functioning in electron transfer. Because of these structural and suggested functional relationships, and also because of their wide distribution and the uncertainty of their exact metabolic roles, vitamins E and K are considered here in this context. Both may have other functions.

a. Vitamins K. There are questionable intermediates in electron transport chains, but they are probably involved in oxidative phosphorylation between DPNH and cytochrome b. Phosphate esters of the reduced form may, on oxidation, transfer phosphate to ADP (*185*). It is possibly significant that in liver virtually all the vitamin K is in the mitochondria. Vitamin K_1 is synthesized by higher plants; vitamin K_2, containing two more isoprene units, is a product of bacteria, and to a very minor degree, of yeasts and molds. Phthiocol (from *Mycobacterium tuberculosis*) contains a hydroxyl group in place of the side chain; and "menadione" is the naphthoquinone portion of the vitamin and does not occur naturally. These latter abbreviated forms of the vitamin are biologically active through *in vivo* attachment of the isoprenoid moiety. Reduced forms are active, and a synthetic imino analog of vitamin K_1 has over four times its biological activity. Menadione (0.01–1.0 μg. per milliliter) and phthiocol (0.01–10 μg. per milliliter) stimulate the growth of Johne's bacillus (*Mycobacterium paratuberculosis*) (*198*), *Lactobacillus bifidus* var. *pennsylvanicus* (*199*), and *Fusiformis nigrescens* (*200*). The latter might serve as a suitable microbiological assay organism.

Vitamin K is produced in sufficient quantities by intestinal bacteria to prevent primary deficiencies in animals, except in the newborn. Conditioned deficiencies may arise, however, through intestinal disturbances which interfere with absorption or bacterial synthesis and through the action of naturally occurring inhibitory analogs such as dicumarol. In vitamin K deficiency, there is an impairment in the hepatic synthesis of proconvertin and prothrombin (for some unknown reason). The only recognized symptom of vitamin K deficiency is a prolonged clotting time. Deficiencies have been induced in most common birds and mammals, but quantitative requirements are not well established because of the generous activities of the intestinal symbionts.

b. Tocopherols (Vitamins E). Tocopherols have been considered as possible intermediates in electron transfer systems, but the present evidence does not favor this possibility (*185*). Tocopherols are known to function as inhibitors of the autoxidation of polyunsaturated fatty acids (PUFA) (*201*), and probably have other specific functions in both plants and animals. The seven different tocopherols that have been isolated from natural sources all differ in the location of methyl groups around the chromane nucleus, have different biological distributions, and have different qualitative and quantitative biological activities. α-Tocopherol is not surpassed in its biological efficacy by any other analog. Methylene blue, thioneine, and a variety of other unnatural substances also alleviate some (but not all) of the symptoms of tocopherol deficiency, as do trace amounts of selenium and its organic derivatives (cf. VI,B,12). Tocopherols occur in both plants and animals as the free alcohols. The corresponding quinones are biologically inactive. Plants synthesize vitamin E in sufficient quantities to meet their needs, and plant germs contain the greatest amounts. Microorganisms contain only traces, and levels are generally low in animals.

Higher animals cannot synthesize tocopherols, and requirements have been reported for insects (*Ephestia*) (*202*), crustaceans (*Daphnia*) (*203*), amphibians (tadpoles) (*204*), fishes (*Lebistes*) (*205*), birds (chicks, ducklings), and mammals (mouse, rat, guinea pig, rabbit, dog, sheep, goat, cow, monkey). There is good recent evidence for a human requirement (*201*) and for the occurrence of human deficiencies; a multitude of earlier claims have been discredited. Sterility is one of the most uniform symptoms of deficiency, but is of different origin in the male and female. Successful bearing of young in the female may require amounts of vitamin E that are ten or more times greater than are required to prevent male sterility. Herbivorous animals seem particularly sensitive to deficiencies and rapidly develop a muscular dystrophy that occurs only much later in other species. Birds manifest encephalomalacia. Concurrent vitamin A deficiencies may develop through failure to protect this from autoxidation. Symptoms of tocopherol deficiency may be due to essential fatty acid deficiency, to the toxic effect of unsaturated fatty acid peroxides, to other concurrent deficiencies, and to the impairment of other unrelated functions of tocopherols. It has been shown that, among different species, there is a 70% increase in the requirement for every 100% increase in body weight (*206*). Comparisons are difficult, however, because the needs are exceedingly dependent upon the levels of polyunsaturated fatty acids in the diet (as are the deficiency symptoms), and requirements may vary from a mere trace to considerable amounts in any given species.

5. *Essential Fatty Acids*

These fatty acids are involved directly in, or are closely associated with, electron transport systems, but their precise function is unknown

(*207*). They have been suggested as mediators in phosphorylations coupled with the oxidation of reduced cytochrome c. It is certain that high concentrations occur in close approximation to cytochrome oxidase, but this has also been interpreted as indicating that the enzymes are attached to lipid membranes which are required for their proper orientation (*208*). The essential fatty acids are also believed to function as vital structural constituents of cellular membranes, and in the transport of other lipids, but these roles may also involve the transfer of electrons. In order for fatty acids to have biological activity they must have the following structural characteristics: (*a*) they must be *cis*-polyenoic acids in which the double bond system is methylene interrupted (not conjugated); and (*b*) they must contain double bonds at least in the 6,7 and 9,10 positions, *counted from the terminal methyl group.* All *in vivo* transformations between these acids in animals are known to occur at the carboxyl group, or between this and the nearest double bond. The divinyl methylene groups are highly reactive and are susceptible to ready autoxidation. These same groups provide the structural characteristics for the specific substrates of the important plant lipoxidases. Arachidonic acid, with four double bonds, is the most unsaturated active form, displays the highest potency, alleviates all deficiency symptoms, and is believed, therefore, to be the functional form. Linoleic, linolenic, and a few other polyenoic acids are believed to function as precursors, and vitamin B_6 is involved in the conversion. Many polyenoic acids *not* possessing all the mentioned structural characteristics occur in nature but are biologically inactive. Polyunsaturated fatty acids, including active ones, are synthesized in quantity by plants, and are widely distributed. They are present in much smaller amounts in animals, but their structures are more varied.

Linoleic acid stimulates the growth of certain lactobacilli and other organisms and is required by an unidentified micrococcus, but it is inhibitory to most bacteria. Oleic acid, which is monounsaturated and inactive in animals, is required by *Corynebacterium diphtheriae* and *Clostridium tetani* (1 μg. per milliliter) and stimulates many other bacteria and fungi (*Pityrosporum ovale* at 0.1–10 mg. per milliliter). Linoleic acid is required by *Trichomonas vaginalis* (*195*). Requirements have been indicated in the sugar beet webworm (*Loxostege sticticalis*) and in various *Ephestia* species (*202*), but not in Tenebrionidae. Deficiencies of essential fatty acids have been observed in many higher animal species (chickens, mice, rats, guinea pigs, hamsters, hogs, primates, etc.), but it is probable that most animals can synthesize *limited* amounts. A great many factors may affect the requirement. Quantitative needs are most satisfactorily expressed in terms of a percentage of the daily calorie consumption. An adequate supply is assured for human infants if about

4% of their calories are in the dietary form of linoleic acid; 1% is the minimum allowance. Essential eczema occurs as a result of dietary inadequacy in human infants. Growing evidence indicates that hyperlipemia and atherosclerosis *may* occur as a consequence of deficiency *relative* to the levels of saturated fatty acids. Atheromas occur in monkeys and rats on a vitamin B_6-deficient diet, in which case arachidonic acid synthesis is impaired. Essential fatty acid deficiency symptoms in other animals include dermatitis, kidney damage, accumulation of liver lipids, and impaired reproduction.

6. *Ascorbic Acid (Vitamin C)*

Ascorbic acid (here abbreviated ASH_2) has been suggested as a component of electron transfer systems in animal tissues (*209*)

$$\mathrm{DPNH/DPN - ASH/ASH_2 - Cyt.\ b_5H/Cyt.\ b_5 - O_2/H_2O} \tag{8}$$

and in green plants,

$$\mathrm{MH/M - TPN/TPNH - GSH/GSSG - AS/ASH_2 - H_2O/O_2} \tag{9}$$

Ascorbic acid oxidase, which is present in plant tissues, but not in animals, may function in the latter system. Both ascorbic acid and dehydroascorbic acid are biologically active. Vitamin C is apparently involved in many metabolic oxidations: in phenylalanine and tyrosine oxidation; in proline oxidation to hydroxyproline (and thus collagen formation); in folinic acid synthesis; in steroid oxidation, etc. It is not a known coenzyme for any specific enzyme system, however, and its action is usually nonspecific. It is synthesized by most plants and animals from glucose via the gluconate oxidative pathway. Ascorbic acid is present in large amounts in some animal endocrine glands and in plant ovaries (fruits and berries), being classically associated with citrate accumulation in citrus fruits. Green leaves also contain large amounts, but it is probable that the major single source in most American and European diets is the potato.

Ascorbic acid is required in the diet of primates, the guinea pig, and some locusts (e.g., *Shistocerca gregaria*) (*210*), but not in other species studied to date. Vitamin C is not known to occur in or be functional in microorganisms to any great extent, although its redox properties may make it beneficial to many. This latter effect may be seen in *Trichomonas*, but its requirement is much more specific in some Trypanosomidae (*195*). Deficiency in higher animals results in scurvy, with attendant symptoms of impaired collagen synthesis. This is most marked in the skeletal system and by increased capillary permeability. Ascorbic acid is believed to play some role in the molting process of locusts.

7. *Glutathione*

Glutathione has been implicated in oxidation-reduction reactions in many biological systems (cf. VI,C,6; VI,J), but most of these are nonspecific. There is evidence also that it functions in additions to carbon-carbon double bonds and to carbonyl groups. It is widely distributed in both plants and animals and is synthesized from its component parts (glutamic acid, cysteine, glycine) (cf. V,A,3). Glutathione is reported to be required in the nutrition of a few microorganisms, but its role is generally nonspecific. A similar consideration holds for its requirement by the brine shrimp *Artemia salina* (cf. VI,G,1). It is specifically required by *Hydra* as a phagostimulant (*172*). Recent evidence indicates that it may have an important functional role with spermine (cf. VI,J).

D. VITAMINS INVOLVED IN CO_2 FORMATION AND FIXATION

1. *Thiamine (Vitamin B_1)*

In the form of the pyrophosphate (cocarboxylase), thiamine functions in the α-decarboxylation of oxalic, pyruvic, and ketoglutaric acids. It does not participate in *all* keto acid α-decarboxylations, and some decarboxylases in which it does function do act on other unnatural substrates (e.g., α-ketobutyric and α-ketovaleric acids). Reaction is by formation of an unstable intermediate between the α-carbon of the acid and the C-2 of the thiamine thiazolium salt (*211*), with subsequent decomposition to yield CO_2 and acetaldehyde in one case, or derivatives of lipoic acid and then CoA in oxidative decarboxylation. The numerous products ascribed to the reaction (*212*) vary with these subsequent events. Thiamine is of universal biological occurrence, being synthesized by most plants but not by any animal studied. It is synthesized from a thiazole and a pyrimidine moiety (cf. V,C,1), but little is known of the origin of either of these two structures. Both of these fragments are biologically inactive, except as thiamine precursors, and thiamine itself is *biologically* inactive, except as its polyphosphate coenzyme form. A great many thiamine, pyrimidine, and thiazole analogs have been tested as to their biological activity (*133*). It is reported that certain naturally occurring pterins may substitute for thiamine in some instances (*213*).

Most plants do not require a nutritional source of thiamine or its components. Portions of higher plants (e.g., roots) may require either the pyrimidine or the thiazole portions of thiamine, or both, but *are* able to connect them; this is also true of many bacteria, yeasts, and molds (cf. V,C,1). For instance, *Mucor ramannianus* requires the thiazole fragment only, and *Rhodotorula rubra* needs only the pyrimidine, so that the two microorganisms can grow together symbiotically in a thiamine-free medium (*214*). Many lower plants cannot connect the fragments, and so require the intact thiamine molecule. This is also true of all animals. A few microorganisms (e.g., *Neisseria gonorrhea*) cannot synthesize the coenzyme, so that thiamine is inactive and there is a nutritional need for cocarboxylase. This is also true of *Borrelia vincentii* (*215*), which in addition requires codecarboxylase, coenzyme A, DPN, and ATP. Thiamine requirements have been demonstrated for many protozoa and for all other invertebrates (nematodes, insects, crustaceans) and vertebrates (fishes, birds, mammals) studied to date.

The quantitative requirement on a weight basis among different species varies inversely with the species weight and is highly dependent upon the carbohydrate content of the diet. Classic deficiency syndromes in man are beriberi and nutritional polyneuritis. Symptoms in all animals include anorexia, impaired growth, cardiovascular disturbances, peripheral neuritis, and gastrointestinal disorders (including achlorhydria). Symptoms vary greatly among species. Mice, for instance, die so rapidly in the complete absence of thiamine that typical symptoms do not develop. Thiamine deficiencies in humans result primarily from heavy consumption of refined flours, from which the thiamine-containing husks of the grain have been removed. Conditioned deficiencies may be caused by thiaminases found in the viscera of many fish and mollusks, and inadvertent inclusion of these in the diets of fox and mink has been responsible for fatal deficiencies in these animals.

2. *Biotin*

Biotin functions in a number of processes in which CO_2 is fixed into larger organic molecules or released from them. These include the β-decarboxylation of polybasic keto acids, and various poorly defined steps involved in amino acid and fatty acid metabolism. Specific coenzymes are unknown, but it is believed that numerous biotin-containing enzymes exist and that the ureido group of these may be activated by ring formation with the side chain. It has been demonstrated that CO_2 combines with the ureido nitrogen of biotin, the vitamin acting as a CO_2 carrier (*216*). Oxybiotin is almost as active as biotin for most organisms; its activity is probably direct, not as a precursor. Desthiobiotin is highly active for certain microorganisms, but not others, and it probably serves as an intermediate in biotin synthesis. Pimelic acid and cystine are apparently the precursors of biotin (*217*). Oleic acid also spares the biotin requirement of some bacteria. Biotin occurs in water-soluble form in most plants, a portion of which is accounted for by *biocytin* (ϵ-*N*-biotinyl-L-lysine). Biocytin is active only for some organisms. Biotin occurs predominantly in water-insoluble form in most animal tissues. It also occurs in nature as biotin 1-sulfoxide (AN factor). Biotin is synthesized by most plants, but required by some parts of plants and by bacteria, yeasts, molds, phytoflagellates, and all animals except *Tetrahymena* studied to date (*218*). The need in insects, fish, birds, and mammals is well established. Intestinal synthesis by symbionts is adequate to supply the minute requirements (less than 1 μg. per kilogram body weight). Deficiencies may be induced by the feeding of avidin, a glycoprotein from egg white that irreversibly binds biotin. Symptoms include dermatitis, alopecia, and muscular dystrophy but are variable among the different species.

E. VITAMINS THAT LABILIZE α-CARBON ATOMS

Vitamin B_6 functions as the 5-phosphate esters of pyridoxal and pyridoxamine (codecarboxylase) in a number of transformations of amino acids and keto acids. The alcohol analog of the vitamin, pyridoxine, also occurs in nature. The reactions of vitamin B_6 all involve the formation of Schiff-type intermediates with the α-carbon atom of the substrate, and subsequent electron shifts in the bonds surrounding the α-carbon, so as to catalyze decarboxylations, transaminations, dehydrations, desulfhydrations, racemizations, serine-indole condensations, etc. Most of these reactions can be induced in simple *in vitro* systems in the absence of the apoenzyme but in the presence of a metal ion which stabilizes the Schiff base by chelation. The role of vitamin B_6 in arachidonate synthesis is as yet unclear (cf. VI,C,5). Thus, the function of this vitamin in amino acid interconversions is of tremendous importance, as indicated by the fact that the *qualitative* amino acid requirements of many bacteria are dependent upon the vitamin B_6 intake and that the *quantitative* requirement for vitamin B_6 in animals varies with the protein content of the diet. The biogenic origin of vitamin B_6 is not known; the three forms are interconvertible in animals and higher plants, but not in some microorganisms. Synthesis of the coenzyme (the phosphate) by pyridoxal kinase is common to all species except a few bacteria and is inhibited by very low concentrations of isoniazid (isonicotinic acid hydrazide).

Vitamin B_6 is not required by entire higher plants, nor by many microorganisms. Of the microbes requiring it, some few respond only to the phosphate (*Lactobacillus acidophilus, L. helveticus*); a few only to pyridoxal (*L. casei*); many to both the aldehyde and amine, but not the alcohol (*L. arabinosis, Streptococcus faecalis, Tetrahymena geleii*); and many to all three forms (*133*). Vitamin B_6 stimulates the growth of some protozoans (*Chilomonas*) and is essential for others (*Tetrahymena, Colpoda*). All animals probably require the vitamin and can apparently use all forms, but as in the case of microorganisms that utilize more than a single analog, the activities are not always equal. Mammalian tissue cultures utilize all three forms about equally, however. Requirements seem assured in the nematodes (*188*). Tapeworms (e.g., *Hymenolepis diminutia*) apparently have a very limited capacity to perform transaminations and utilize synthesis only to a minor extent in meeting amino acid requirements (*219*), a fact suggesting a low vitamin B_6 requirement. Balanced against this is their great amino acid absorbing capacity; vitamin B_6 has been suggested as important in active transport of amino acids across cell membranes. The intracellular symbionts of some insects, such as *Stegobium paniceum* and *Lasioderma serricorne*, synthesize most of the required accessory growth factors for the host, but in these two insects, adequate amounts of vitamin B_6 (and thiamine) are not provided. In other instances (*Pseudosarcophaga affinis*) the symbiotic supply is adequate, and dietary requirements are difficult to demonstrate. Rice moth larvae (*Corcyra cephalonica*) manifest symptoms of deficiency similar to those seen in vertebrates (*114*), including failure of hydroxykynurenine conversion to hydroxyanthranilate, and consequent failure of niacin synthesis. Deficiencies have been induced in fishes, birds, and mammals, and requirements have been evaluated, being in all cases dependent upon the dietary protein levels. Deficiencies have been observed in human infants ingesting a commerical infant-feeding fomula and in dairy cattle, but classic naturally occurring deficiencies are not known in animals.

F. Vitamins That Transfer Acyl Groups

1. *Pantothenic Acid*

Pantothenic acid functions as a part of coenzyme A, in which it is coupled to β-mercaptoethylamine and 3′-phosphoadenosine diphosphate. In this form it is involved in the transport of acetyl and other acyl derivatives between thiamine, lipoic acid, and various metabolic intermediates in many important steps in carbohydrate and lipid metabolism. Pantothenic acid is synthesized from β-alanine and pantoic acid, the former arising from aspartate and the latter from α-ketoisovalerate, the precursor of valine (cf. V,A,6). The β-mercaptoethylamine fragment of CoA arises from cysteine via pantothenylcysteine and its decarboxylation product, pantetheine. Most plants can synthesize pantothenic acid, but a few microorganisms are known which require a nutritional source even of CoA. Some higher plant parts require only one or the other structural portion of pantothenic acid, being able to synthesize the other.

Animals uniformly seem to require a dietary source of both parts *combined,* a mixture of β-alanine and pantoic acid being inactive. This appears of necessity, since the valine precursor is not available in animals (cf. V,A,6). Intestinal microbial synthesis is extensive in most animals, but digestive cleavage of the peptide bond of pantothenate is negligible; naturally occurring deficiencies are thus rare. Requirements have been verified in nematodes (*188*), in insects (*151*), and in crustaceans (*Daphnia*) (*220*). Deficiencies have been induced in many higher animals (fishes, birds, and mammals). Symptoms are most pronounced in the epidermal tissues, occurring to a lesser extent in the gastrointestinal and nervous systems. Symptoms are quite species specific. It is of some interest that the herbicide 2,3-dichloroisobutyrate inhibits pantothenate synthesis in those species of plants in which it has been studied.

2. *Lipoic Acid (6,8-Dithiooctanoic Acid)*

This acid functions, as the cocarboxylase conjugate (lipothiamide), in the transfer of acyl groups between thiamine pyrophosphate and coenzyme A in the oxidative decarboxylation of pyruvate and α-ketoglutarate (*221*). It may function also in other analogous systems and in the oxidation of sulfite to sulfate. It has been suggested as a primary hydrogen acceptor in photosynthesis, and it transfers electrons to DPN^+ in oxidative decarboxylations. Lipoic acid is widely distributed in nature, but is required nutritionally by only a few species as far as is known. Among these are various *Escherichia coli* mutants, *Streptococcus cremoris, S. faecalis* (for pyruvate oxidation), *Butyribacterium rettgeri, Lactobacillus casei,* and *Tetrahymena geleii*). Its requirement by the latter protozoan occasioned its earlier name, protogen. In the onion maggot, *Hylemya antiqua,* it is required only by the female of the species (*114*), which also appears to require vitamin B_{12} and coenzyme A. It seems possible that lipoic acid will appear as a more general nutritional requirement in future investigations.

G. VITAMINS THAT TRANSFER SINGLE-CARBON UNITS

1. *Folic Acid (Pteroylglutamic Acid)*

Folic acid and its various polyglutamate conjugates function in the form of folinic acid (citrovorum factor, leucovorin, 5-formyl-5,6,7,8-tetrahydrofolic acid) in the transfer of single-carbon fragments in numerous metabolic sequences, including purine and pyrimidine synthesis. For this latter reason, adenine and thymine eliminate the nutritional requirement for folic acid in *Streptococcus faecalis,* and cells grown in this fashion contain no detectable amount of folic acid, but have normal quantities of DNA. Folic acid polyglutamyl conjugates are widely distributed in nature and are converted to folic acid in the tissues prior to utilization. Folic acid is synthesized from its pteridine, glutamic acid, and *p*-aminobenzoic acid. The pteridine of folic acid is closely related to a large number of closely related, naturally occurring pteridines (*222*), which seem to have common biogenic origins with flavins (riboflavin) and purines (*223*). Xanthopterin, isolated from butterfly wings and found in many other sources, replaces folic acid in the nutrition of many animal species. It has antianemic properties in the monkey, but not the chick or rat. Biopterin from human urine and from beef liver is active for the trypanosome *Crithidia fasciculata* and for various other species. Rhizopterin (formylpteroic acid), from *Rhizopus nigricans*, is highly active for some species (*Streptococcus lactis*), but not for others (*Lactobacillus casei*). Insects contain a particularly varied collection of pterins. The varying biological activity of some pterins suggests that many animals may have slight folic acid-synthesizing capabilities.

While higher plants and most bacteria can synthesize folic acid, a few bacteria cannot. In view of the previous discussion, it is apparent that a number of substances may modify substantially the folic acid requirements of different plants and animals, because (*a*) they may be folic acid precursors (pterins), (*b*) they are products of reactions catalyzed by folic acid (purines), and (*c*) they stimulate the achievement of proper folinic acid levels (ascorbic acid). Thus, *Tetrahymena geleii* requires folic acid (0.65 $\mu\mu$g. per milliliter), but can utilize 500-fold amounts of rhizopterin. The purine replacement of folic acid requirements has already been mentioned. Finally, as in (*c*) above, folic acid-deficient diets lead to megaloblastic anemia in monkeys, but only when vitamin C levels are also inadequate.

Folic acid is required by the nematode *Caenorhabditis briggsae* (*224*) and is apparently spared somewhat by glutamine, but not replaced by biopterin. Folic acid is required by the brine shrimp *Artemia salina* (*225*) and also by insects. Deficiency symptoms vary among the species, but include macrocytic anemias, leucopenia, growth failure, and gastrointestinal and other disorders. Deficiency diets are not effective in some species unless modified by the incorporation of sulfonamides or unless folic acid antagonists are employed. Inhibitory analogs have also been of some value in the treatment of neoplastic diseases. The human macrocytic anemias of sprue, pregnancy, and infancy often respond to folic acid, and sometimes to thymidine. Reticulocyte responses are obtained also in pernicious anemia (cf. VI,G,3), but in this instance vitamin B_{12} is required to alleviate the neurological syndrome.

2. *p-Aminobenzoic Acid* (PABA)

In its various forms PABA is widely distributed in nature and apparently has as its sole function its role as a precursor of the folic acid molecule. In *Lactobacillus arabinosus,* the synthesis of folic acid is exactly proportional to the PABA content of the medium. There are still some reasons for believing in the existence of other PABA functions, however. Folic acid, for instance, is not always active as a PABA source for bacterial growth and has been shown to be inactive in *Escherichia coli* and *Neurospora* PABA-less mutants. Methionine is active in the latter case, and these data, among others, suggest alternate roles for PABA function. PABA is apparently synthesized by higher plants and by most microorganisms from shikimic acid, but it is required by several dozen known bacterial species in minute amounts (0.03–1.0 $\mu\mu$g. per milliliter (*226*). Deficiencies induced in higher animals (mouse, rat, dog, fox) are manifest by graying of hair, but PABA deficiency has not been clearly associated with this symptom in man. Growth is generally depressed in chicks, and fatty livers occur in deficient trout. PABA reverses the sulfonamide inhibition of folic acid synthesis in bacteria. At tremendous levels in the diet (3% for mice), it is highly effective against certain rickettsial diseases and is used therapeutically for that purpose.

3. *Vitamin B_{12}*

This vitamin (known also as cobalamin, zoopherin, animal protein factor) exists in various forms in nature; the forms vary according to whether it is a cyanocobalamin (the usual active form), nitrocobalamin, hydroxycobalamin ($B_{12a,b}$), sulfitocobalamin, etc. Its natural 5,6-dimethylbenzimidazole moiety may be replaced in the pseudovitamins B_{12} by adenine, 2-methyladenine, 5-hydroxybenzimidazole, etc. A number of coenzymes have been isolated. Adenylcobamide (AC) from *Clostridium tetanomorphum* has an adenine in place of the benzimidazole moiety (pseudovitamin B_{12}), and another adenine-sugar group in place of the usual cobalt-bound cyanide of cyanocobalamin (*227*). This coenzyme catalyzes the conversion of glutamate to β-methylaspartate; it is as active for an *Escherichia coli* mutant as is cyanocobalamin, and three times as active as the pseudovitamin B_{12} from which it is derived. It is completely inactive for *Ochromonas malhamensis,* which responds to cobalamin, but not the pseudovitamin. A second coenzyme, dimethylbenzimidazolylcobamide (DBC) has been isolated from *Propionibacterium shermanii* and is a derivative of cobalamin proper (*228*). A third coenzyme, formed by *Clostridium tetanomorphum,* grown in the presence of benzimidazole, is known as benzimidazolylcobamide (BC). All the cobamide coenzymes thus appear to be cyanide-replaced, adenine-sugar derivatives of various vitamin B_{12} analogs (*229*). The activities of the various cobalamin derivatives vary from species to species, but the pseudocobalamins are apparently inactive for most animals.

Vitamin B_{12} functions in (*a*) the reduction of, and (*b*) the transfer of single-carbon fragments (*230*), and possibly in other as yet unknown reactions. In this way it is intimately involved in amino acid and nucleic acid metabolism and in electron transfer reactions involving thio groups (*231*). The major route of glutamate metabolism in *C. tetanomorphum* is via β-methylaspartate to mesaconate (methyl fumarate), a shunt that differs greatly from the tricarboxylic acid cycle. The role of β-methylaspartate in animal metabolism in unknown, but this amino acid is reported to cause reticulocytosis and general improvement in pernicious anemia patients, with no increase in hemoglobin, when administered at levels of 1 gm. per day (*232*). In general, it is apparent that the metabolic role of cobalamin and its derivatives is still but poorly defined.

Higher plants uniquely appear to require a nutritional source of vitamin B_{12}, and they do not concentrate sources that are available to them, as do animals, so that cobalamin levels in plants are scarcely detectable. Vitamin B_{12} must be provided to plants by soil bacteria, and plant roots are thus the richest portion (about 0.01 μg. per gram fresh material); stems have less (about 0.0005 μg. per gram), and in leaves the vitamin is scarcely detectable (<0.02 mμg. per gram). Roots of plants grown in synthetic mineral solutions contain scarcely detectable amounts. Animals (insects, birds, fishes, and mammals) also require vitamin B_{12}, but their tissues contain it in much greater amounts. Vitamin B_{12} deficiencies are known to occur in individuals who consume no animal products, the blood levels dropping in these cases from the normal range of 200–350 μμg. per milliliter to 40–200 μμg. The widespread distribution of vitamin B_{12}, its vigorous bacterial biosynthesis in the gastrointestinal tract, and its very high biological activity tend, however, to preclude deficiencies. The requirement is only about 1 μg. per day in humans and about 10 μμg. per gram of diet for other animal species. Cobalamin supports the half-maximum growth of *Lactobacillus lactis* Dorner at levels of 13 μμg. per milliliter of medium. Apparently the sole biological source of vitamin B_{12} is microbial synthesis, and most bacteria produce it in quantity. As a consequence, *Streptomyces griseus* and *Bacillus megatherium* fermentations are the major commercial source; activated sewage sludge contains up to 50 μg. per gram, and manure (dry) about 0.5 μg. per gram. From among the several microorganisms in which a requirement exists, several species have been selected as assay organisms, including *L. lactis* Dorner, *L. leichmannii*

(7830 and 4797), *Euglena gracilis. Ochromonas malhamensis,* and various *Escherichia coli* mutants. Assays have also been devised with chicks and rats.

Of some ninety species of marine flagellates and algae for which the nutritional requirements are known, 33% required no vitamins whatsoever, 50% required vitamin B_{12}, and half of these required nothing else of an organic nature; 40% required thiamine. Five species required biotin, and one each required riboflavin, glycine, and histidine (*149*). Among these species, less than one-third of the predominantly vegetal Chlorophyceae and Bacillariophyceae require vitamin B_{12}, while in the more saprophytic or holozoic Euglenineae, Cryptophyceae, Dinophyceae, and Chrysophyceae over 90% had a B_{12} requirement. Provasoli (*233*) has pointed out that this stereotyped pattern of organic requirements (only three vitamins) is too strange to be fortuitous and that it may represent a phylogenetic characteristic of the algae, especially those of the chlorophyte-leucocyte transition. The trends for vitamin B_{12} requirements among these groups reflect the traditional plant-animal dichotomy of vitamin biosynthesis, and vitamin B_{12} deficiencies have not been actually *demonstrated* in higher plants. It is thus tempting to wonder whether the low levels in plant leaves may not reflect a very high demand for catalysis of single carbon addition to ribulose during photosynthesis (cf. IV,A).

Vitamin B_{12} deficiencies in humans often result from a conditioned deficiency due to the failure of absorption, which normally requires the intermediation of a gastric juice "intrinsic factor." This factor is constitutionally absent in some individuals, resulting in a "pernicious anemia" which is characterized by macrocytic anemia and eventual neurological involvement. Either folic acid or vitamin B_{12} are effective in treatment of the anemia, but B_{12} alone brings about a neurological response. Deficiency of cobalamin produces retarded growth in all animals studied. The effect of vitamin B_{12} in promoting growth in poultry, rats, pigs, and ruminants is enhanced by addition to the diet of certain antibiotics. Vitamin B_{12} supplements are apparently without effect in normal children, but they increase the weight of underweight children and increase the height of the overweight, establishing more normal height: weight ratios.

4. *Choline*

Choline and its derivatives function in the transfer and storage of "labile" methyl groups in intermediary metabolism. It is synthesized from serine-derived ethanolamine and from methyl groups primarily derived from methionine (a fraction is of endogenous origin). The derivatives of choline include ethanolamine, mono- and dimethyl derivatives, phospholipids, betaines, and thetins (sulfonium analogs of betaine). In animals possessing choline oxidase, choline furnishes methyl groups to form methionine (cf. V,A,8). In other animals (rabbits, chicks, guinea pigs), betaines or thetins are methyl donors. Choline has a second function as a structural component of phospholipids and is thus essential for the mobilization of fats, particularly from the liver. Its dipolar nature is of great importance in maintaining the polarity of cell membranes, and this is emphasized in the case of acetylcholine. The synthesis of choline appears to occur in all species in which there is an adequate source of labile methyl groups, but the rate may not meet the biological need, and genetic limitations may occur in some instances. Choline *requirements* are thus not absolute. Only catalytic amounts of choline may be required for methyl group transfer; the question of whether or not choline is a *vitamin* is dialectic, and there is no doubt that large amounts of choline may be required for other functions in some species.

Choline is not required nutritionally by higher plants, and is a nutritional need for only a few bacteria (*Diplococcus pneumoniae* types I, II, III, V, VIII). *Neurospora crassa* mutants have been obtained which are blocked at each of the biosynthetic methylation steps and which respond to as little as 0.02 μg. per milliliter. In this case (and in animals) there are specific methyl donors for each step, and no two are precisely equivalent. Among the protozoa, choline is not required by *Tetrahymena*, but *Crithidia fasciculata* requires a methyl donor of some sort. Requirements for animals are exceedingly dependent upon the methionine level, and upon vitamin B_{12} and folic acid because of methyl group synthesis. Choline is apparently required by all insects. For *Phormia regina* (blowfly), either dimethylethanolamine or carnitine will replace choline, but not betaine. Carnitine (cf. VI,G,5) also partially replaces choline for *Drosophila*, but not for *Blattella germanica* (which has a very high requirement) and many other species.

Choline, or a labile methyl source, is required by all of the fishes, birds, and mammals studied to date, but deficiencies do not occur except on low protein (methyl donor) diets. Choline is required in mammalian tissue culture. Perosis (cf. VI,B,9) is the classic deficiency symptom in chicks and poults, but neither betaine nor methionine are effective in alleviating this symptom. Fatty infiltration of the liver and hemorrhagic kidneys are major deficiency symptoms in all animals. All labile methyl compounds are lipotropic and antihemorrhagic, but not with equal efficacy. Requirements are higher than for most other "vitamins," being of the order of 50–100 mg. per kilogram body weight for most species, but highly variable because of the factors mentioned and because of species differences. Requirements are highest in the young; as in the case of arginine (cf. V,A,11), the synthetic rate is apparently adequate to meet adult needs on an otherwise satisfactory diet.

5. *Carnitine (Vitamin B_T) (β-Hydroxy-γ-trimethylaminobutyric Acid)*

This vitamin functions as a methyl donor in a few insects (cf. VI,G,4), but not in most others, nor in birds or rodents. It may function in acetyl transfer, since an enzyme found in pigeon liver catalyzes its *O*-acetylation by acetyl CoA; and the acetyl derivative, in the presence of CoA, can acetylate *p*-aminobenzoate or sulfonamides (*114*). Carnitine occurs in muscle tissue and is probably derived from β-hydroxy-γ-aminobutyric acid. The latter substance is an oxidation product of γ-aminobutyric acid, which occurs in both plants and animals and is formed by the pyridoxal-catalyzed decarboxylation of glutamate. γ-Aminobutyric acid is believed to play an important metabolic role in both brain tissue and plants. Carnitine is a nutritional requirement, so far as is known, only for several beetle larvae of the family Tenebrionidae, a few closely related species, and probably *Aedes aegypti* (*151*). It is not a nutritional requirement for most insects, nor for other animals, although it is synthesized by other animals and probably fulfills a functional requirement in them. The nutritional requirement may not be as exclusive as it appears, however, since the intestinal microflora of Tenebrionidae contribute very little toward their vitamin supplies, as compared with other insects. Carnitine-deficient *Tenebrio* larvae do not mobilize depot fat; in this regard carnitine resembles choline. The larvae also die at the molt, a phenomenon suggesting failure to produce and transfer two-carbon fragments necessary for new cuticle formation. In the presence of 0.35 μg. of carnitine per gram of diet adults are formed, but these are misshapen and not viable. The nutritional requirement is about 0.75 μg. per gram of diet. β-Hydroxy-γ-aminobutyric acid is active in fifteen- to thirtyfold amounts, but anhydrocarnitine is inactive, suggesting the need for the acetylable hydroxyl group.

H. Carotenoid Vitamins

Vitamin A is the basis for all known photoreceptive systems in higher animals, but is not known to function in the diffuse light sensitivity of the lower invertebrates (*234*). It also functions, in vertebrates at least, in some other critical processes in tissue metabolism which have long resisted elucidation. It occurs in two major forms, vitamin A_1 and vitamin A_2, along with their aldehydes (retinenes) in visual systems and fatty acid esters in liver storage forms. Vitamin A_1 contains a cyclocitral group, vitamin A_2 a dehydrocyclocitral group. Vitamin A_2 absorbs in a somewhat longer wavelength range than vitamin A_1, an observation of considerable phylogenetic significance (*235*). Vitamin A_1 is almost the sole form in most land animals and marine fishes. Most reptiles have vitamin A_1, but some freshwater turtles have vitamin A_2 entirely. Vitamin A_2 occurs in freshwater fishes, and A_2 is mixed with A_1 in some transitional forms, such as amphibians, lampreys, and euryhaline fishes. Of the latter, fishes maturing in fresh water and migrating seaward (catadromous, such as the "freshwater" eel) have a preponderance of vitamin A_1; those maturing in the sea and migrating to fresh water (anadromous, like the salmon) have a preponderance of the freshwater form (A_2). The occurrence of vitamin A_2 in the primitive lampreys suggests this to be the more ancient form, and vertebrates are believed to have arisen from freshwater forms. Although only vitamin A_1 has been found in invertebrate eyes, it must be noted that the arthropod, cephalopod, and vertebrate eyes have evolved independently, have no anatomical, embryological, or phylogenetic relationships to each other, and may represent equivalent stages of independent evolution in these three instances. In the lamprey, the ratio of vitamin A_1 to A_2 varies greatly with whether the animal is migrating upstream (favoring A_2) or downstream (A_1). There is thus a biochemical morphosis related to the life cycle, and this is even more striking in the amphibia as they pass through different developmental stages.

Vitamin A does not occur in plants and has no known function in them. It must be synthesized by animals from a certain few plant carotenoids which have the appropriate structures for cleavage to vitamin A. For mammals, the known provitamins A are: the widely occurring α-, β-, and γ-carotenes; cryptoxanthin from higher plants; aphanin, aphanicin, and myxoxanthin from certain algae; echinone from specific fungi; torularhodin from a red yeast; and perhaps leprotene from *Mycobacterium phlei*. Carotenoids are not active and have no known vital function in animals other than as provitamins A, but they may be stored and be the basis of pigmentation in some species. The carotenoids have photoreceptive and possibly other functions in plants and are synthesized by them via branched chain intermediates which are also involved in steroid synthesis (cf. VI,I) (*101*). Mevalonic acid (β-hydroxy-β-methyl-δ-valerolactone) (hiochic acid) is the source of the basic, repeating isoprene unit of carotenoids and is a growth factor for *Lactobacillus heterohiochii* (*236*). Some invertebrates (e.g., crustaceans) have species-specific and highly characteristic carotenoids and thus are able to perform limited interconversions among carotenoids, but animals are not believed to synthesize them *de novo*. By such interconversions, fish and invertebrates (and possibly other vertebrates) may be able to enjoy the advantage of a wider range of provitamins than those mentioned for mammals. The conversion of provitamin to vitamin A occurs predominantly in the intestines, and perhaps to a very limited extent in the liver and some other tissues. The mechanism is unknown; apparently the rate is variable and very slow in some animals (*237*); it does not occur at all in some carnivores such as the cat (*238*). Carotene is converted directly to vitamin A_1 in the cod and lobster and to A_2 in the perch and dace; the two forms are not known to be interconvertible in species containing them both, but both are active in all species, regardless of whether they are indigenous to that species. Vitamin A is oxidized to retinene by retinene reductase with the participation of DPN.

There is no known nutritional requirement for carotenoids or vitamin A in plants. These substances are not known to be required by lower invertebrates and are apparently absent from many of them. Many invertebrates have been cultured in media completely devoid of carotenoids and vitamin A, and this is particularly true of the protozoa. The carotenoid requirements for pigmentation in invertebrates, and the obligate nature of such pigmentation, is unknown. Among the mollusks, vitamin A has been suggested as beneficial for the snail *Helix pomatia* (*239*) and occurs in the eye of cephalopods. In this latter, it is not known whether the vitamin A is synthesized from subcarotenoid fragments, converted from requisite carotenoids, or obtained nutritionally. The crustacea utilize vitamin A for photoreception; some of this is known to come to them preformed, and some of it is formed by them from provitamins. The list of provitamins is probably much longer for these species. Vitamin A activity, as measured by rat assay, is very unevenly distributed among crustaceans, suggesting synthesis from astaxanthin and other carotenoids not active as precursors in mammals. Synthesis from subcarotenoid fragments is improbable in crustaceans. Vitamin A or carotenoids are not required in the diet of the isopod *Armadillidium* (the pillbug), but they may arise through intestinal synthesis (*240*).

In insects, vitamin A or carotenoids have not been found to be general nutritional requirements, and have definitely been excluded as such for a great many species. Moreover, vitamin A is apparently absent from most insects. The only exception at present is the desert locust (*Shistocerca gregaria*), in which exclusion of vitamin A from the diet results in retarded growth and only 10% survival to the fifth instar (*241*). It is believed that insect carotenoids are always derived from plant foods (*242*) and that they cannot be converted to vitamin A. Conversion of provitamin to vitamin A has been reported in the silkworm (*243*), however, and retinene$_1$ has been reported as the visual principle in the eye of the bee (*244*) and housefly (*235*) (and probably is in other insects).

In arthropods and mollusks, then, vitamin A is probably required functionally; it may be obtained in the diet, or from carotenoids in some cases. In the latter instance, carotenoids are chemical needs which must be supplied by dietary means or through intestinal synthesis.

Vitamin A, or provitamins A for many species, are chemical needs for all vertebrates that have been studied. In the fifteen or more mammals that have been investigated, vitamin A is required for growth at a uniform level of about 4 μg. (20 I.U.) per kilogram of body weight. Requirements are somewhat higher for successful reproduction, and are also slightly higher for birds (*245*). Deficiencies of vitamin A may be nutritional or conditioned. Impaired digestion or absorption may result from various digestive system diseases or from the injudicious use of mineral oil. Liver disease may impair the ability to convert carotenoids to vitamin A and cause high plasma carotenoid levels. Ocular symptoms include bitot spots on the conjunctival membrane, poor dark adaptation, xerophthalmia, keratomalacia, photophobia, and conjunctivitis. Changes occur also in epithelial tissues, in bone and tooth formation, in reproductive and lactative systems, in growth, and in general health. Prolonged deficiency in cattle causes generalized edema, and permanent total blindness. The vitamin A and carotenoid requirements of domestic birds and of livestock are extensively reported in the agricultural literature.

I. Steroid Vitamins

The basic chemical properties of the steroid nucleus which give it biological value are not known, and the precise biochemical role of no steroid substance has ever been defined. Steroids are synthesized by both plants and animals from valine (cf. V,A,6) via mevalonic acid (cf. VI,H) and squalene, as are carotenoids. There is a great deal of molecular specificity in the steroids synthesized by various species (*246*), and this fact is of taxonomic importance (*247*, *248*). Most prominent among the phytosterols are ergosterol, sitosterol, dehydrositosterol, and stigmasterol. The zoosterols include cholesterol, coprosterol, and cholestanol. In the animal kingdom, the capacity to synthesize specific sterols has been lost in certain species. Plants synthesize sterols, but there are no indications of nutritional requirements in any case. Bacteria and blue-green algae contain only minute amounts, if any at all, while yeasts, molds, and higher plants contain large quantities. In recent years, the fungi have become valuable synthetic tools for the performance of specific steps in steroid chemistry.

Protozoa, and particularly the parasitic species, often require a nutritional source of steroids. *Paramecium aurelia* responds to 10 μg. of sitosterol or stigmasterol, but not to cholesterol or ergosterol (*249*).

Labyrinthula vitellina responds to cholesterol or sitosterol, but not to stigmasterol or ergosterol. *Trichomonas columbae* responds only to cholesterol or closely related compounds (*195*). Cholesterol is active for *Peranema trichophorum* and *Entamoeba histolytica*, and is the most generally available of all the sterols that have been tested. It has also been reported as a requirement for the snail *Helix pomatia*, but little is known of the steroid requirements of the intermediate phyla. Sterols are required by every insect studied in this regard (*114*), and cholesterol or closely related compounds are always active. Most insects can utilize either plant or animal steroids, but the hide beetle, *Dermestes vulpinus*, which is a carnivore, can use only zoosterols (*14*). This is not true of *Phormia regina*, which is also a carnivore but can use phytosterols (*250*). 7-Dehydrocholesterol is active; endocrine steroids and vitamins D are generally inactive for insects, as they are for protozoa. Sterol requirements are in the range of 1–10 μg. per gram of diet. Earlier reports that sterol synthesis is interrupted at the squalene stage have not been confirmed (*251*), but it would appear that multiple synthetic blocks occur. Sterols that inhibit cholesterol esterase thereby prevent cholesterol utilization, indicating the necessity of esterification for absorption (*252*).

The only instance of a demonstrated sterol requirement in vertebrates is the study of a deficiency syndrome in guinea pigs, characterized by stiffness of the wrists, widespread calcium phosphate deposition throughout the body, decreased serum phosphatase, and eventual emaciation and death (*253*). The "antistiffness factor" activity of various natural extracts was thought to be due to sterols, which were found to be curative for the condition.

Activation of alkaline phosphatase, efficient absorption of calcium and phosphate, augmented renal phosphate resorption, and proper mineralization of calcified tissues in vertebrates (and perhaps invertebrates) requires the vitamins D. These substances are derived from the ultraviolet-induced 9,10- cleavage of certain steroid molecules (e.g., ergosterol and 7-dehydrocholesterol, the provitamins D_2 and D_3, respectively). The active forms of the vitamin are thus not steroids. A large number of analogs exist, having varying activities for different species. Thus, vitamin D_2 (calciferol) is less active in the chick than in the rat, but D_3 is equally active in both. The source of the high vitamin D levels in fish liver oils is unknown, since plant and animal plankton do not contain more than traces. Requirements for vitamin D have been demonstrated in all vertebrates studied in this regard, but are difficult of quantitation because of the many factors that influence them. Ultraviolet irradiation of provitamin D_3 in the skin provides adequate supplies of the vitamin in white persons who are exposed to sunlight, and there is thus no nutritional requirement. Licking of the pelt by fur-bearing mammals, and ingestion of feather and uropygial lipids by birds, permits assimilation of activated endogenous provitamins in these species. The classic deficiency syndrome is rickets (osteomalacia in adults); it is primarily manifest by calcification deficiencies and skeletal deformities. Rickets may also be caused by disturbances in parathyroid or calcium physiology (cf. V,D).

J. VITAMINS WITH UNCERTAIN FUNCTIONS

Inositol (*myo*-inositol) is a component of inositol phosphatides and is believed to be required for that reason. It has unexplained lipotropic activity for animals on diets low in protein and free of lipids. It provides a storage form of its calcium and magnesium phosphates (phytates) in seed grains and in avian erythrocytes. Inositides are ubiquitous. Inositol occurs in quantity in mammalian cardiac muscle, and in shark skeletal muscle it may actually have a glycogen-like reserve function. It is apparently synthesized from glucose in mammalian tissues, but deficiencies nevertheless occur, and tissue cultures are reported to require it. It is an established requirement for a number of bacteria, yeasts, and molds (*254*), and inositol-requiring strains of *Neurospora* have been obtained. The need has been demonstrated in only a few insects (*210*). Deficiencies have been observed in mice, rats, guinea pigs, hamsters, chicks, poults, pigs, and humans. Symptoms include alopecia in mice and circumocular alopecia in rats. Inositol prevents the encephalomalacia and exudative diathesis of vitamin E-deficient chicks. Quantitative requirements are much higher than for other vitamins, and it has been pointed out that inositol is the only B vitamin for which the total concentration does not parallel the chromosome size among various animal tissues (*255*). There is no very good reason for thinking of inositol as a vitamin; it appears more likely to be a structural component of vital phospholipids, and specific phospholipases have been found for inositol-containing phospholipids (*256*).

Spermine (III) and spermidine (IV) are widely distributed in nature and are essential to the growth of *Hemophilus parainfluenzae* (*257*).

$$NH_2CH_2CH_2CH_2NH{\cdot}CH_2CH_2CH_2CH_2NH{\cdot}CH_2CH_2CH_2NH_2$$

(III)

$$NH_2CH_2CH_2CH_2NH{\cdot}CH_2CH_2CH_2CH_2NH_2$$

(IV)

Neisseria perflava, Pasteurella tularensis, Lactobacillus casei, and other bacteria on a synthetic medium at concentrations of about 1 μg. per milliliter. Putrescine, cadaverine, and other natural polyamines are only one-thousandth as active. Spermine is synthesized by the condensation of ornithine-derived putrescine with the decarboxylated carbon chain of methionine. *Escherichia coli* contains spermidine, glutathione, and a conjugate of these; a number of observations suggest that the conjugate may have broad biological significance in cell structure: (*a*) rat liver mitrochondria studies indicate that a reaction occurs between the polyamines and a membrane component of the mitochondria; (*b*) spermine inhibits the lysozyme-EDTA-induced lysis of *E. coli;* (*c*) spermine and spermidine have a stabilizing effect on the protoplasts of *E. coli* and bacteriophage T_5; and (*d*) spermine stabilizes a DNA preparation, the *Bacillus subtilis* transforming principle.

Strepogenin is one of a number of substances which have been studied in an effort to determine the cause of the superior growth-promoting properties of hydrolyzed casein over defined media for certain lactobacilli. It is a peptide-like substance which stimulates the growth of certain hemolytic streptococci, *Lactobacillus casei* and mice (258).

Vitamin P (citrinin, hesperidin) consists of a series of flavone glucosides which occur in conjunction with ascorbic acid in plants and which seem to potentiate its antihemorrhagic activity.

Anti-gizzard-erosion factor is a fat-soluble, thermolabile factor found in both plant and animal tissues which prevents the erosion in the gizzard lining of chicks presumably caused by a lack of bile.

Less well known, but perhaps as important, are a vast number of growth factors, for one or another species, that appear in the literature each year. It is impossible to evaluate these early reports in most cases, and only continuing efforts substantiate the preliminary reports and provide functional details. Summaries of these appear in the literature (259).

VII. Summary and Conclusions

The investigation of the biochemical basis of chemical needs throughout the broad range of the plant and animal kingdoms provides a valuable approach to the understanding of the intimate details of metabolic processes. Comparative nutrition, however, is in its infancy, a fact indicated by the scant number of species that have been investigated. To a considerable extent this is due to the tremendous technical problems that arise in culturing most species in the axenic state and in chemically defined media. As a result, many data suggest that our concepts of nutrition reflect more the present status of our knowledge than the actual facts regarding chemical requirements.

The biochemical form of life on this planet developed as a consequence of the chemical and physical conditions prevailing here at the time that the temperature, moisture, and oxidation state of the earth's surface became compatible with the accumulation of complex abiogenic organic materials. Original moisture condensates (seas) were supplemented by simple organic materials and by cations dissolved from the earth's surface and anions distilled from its interior. Biochemical evolution reflects the fitness of this chemical environment to meet chemical needs for the self-sustaining reactions identified with life processes. Nutritional requirements may logically be interpreted in terms of biological needs. The original biopoietic process may have been repeated many times on this planet, and conditions are such as to suggest that similar events have occurred on many other bodies in extraterrestrial space.

Chemical needs have evolved for the maintenance of suitable interrelationships between organisms and their environment through the establishment of osmotic and hydrogen ion defenses. In plants, the osmotically active materials are largely organic, thus providing a degree of independence from the biosphere. In animals, however, aboriginal inorganic electrolytes still predominate, but in some invertebrates the chemical need for these is lessened by the concurrent presence of endogenous organic substances. This is particularly true in the elasmobranchs and in the insects.

The free energy for the formation of oxygen and reduced organic compounds on the earth was originally derived from the direct photolysis of water. In photosynthetic plants, this same process continues, although it is modified to facilitate photon capture at the wavelengths of light penetrating the present atmosphere. Free energy requirements of some lower plants, the chemosynthetic autotrophs, are met by the oxidation of inorganic substances. A class of organisms evolved which had the ability to utilize the products of autotrophic nutrition as a source of free energy, and most of these heterotrophs developed the ability to ingest extraneous materials in particulate form. From the heterotrophs there thus evolved the animals, with their greater dependence upon the biotic environment. Progressively decreasing synthetic capacities, and thus increased nutritional needs, appeared as the result of the ability to circumvent otherwise-lethal mutations by the use of nutritional sources of products of blocked biosynthetic sequences. Only a few highly parasitic animals, however, acquired requirements for specific organic free energy sources. Energy transfer systems of several types developed, employing a variety of electron transport devices and phylogenetically specific phosphagens.

Chemical needs for the provision of morphological structure arose in several ways. Amino acid polymers were employed universally as structural materials. Specific amino acid requirements evolved in certain phyla, depending upon biological needs and the chemical supplies available to the species. A number of amino acid requirements, however, are common to all animal species. Nucleotides arose as components of nucleic acids in all organisms, but biosynthetic capacities seem to be least in primitive species, and chemical needs thus appear to decrease with advancing plant and animal evolution. Carbohydrates serve structural purposes in plants and to some extent in a few prochordates. Mineral structures in lower plants and animals are relatively simple silicates and carbonates, whereas in more specialized species, complex and highly organized phosphocarbonates evolved to give added beneficial properties.

Catalysis of organic transformations was of an inorganic nature in primordial systems, but these simple devices subsequently became more efficient by modification to include reaction-specific proteins (enzymes), and organic functional analogs of the trace elements (coenzymes). The need for some elements, such as sodium, calcium, and chlorine, may have been lost in highly synthetic species (plants), although trace sodium needs appear to remain as a phylogenetic characteristic of the blue-green algae. All the halogens are beneficial to certain species, but none is apparently required universally. Trace element requirements may be phylogenetic characteristics in some biological groups, and the high levels of vanadium and associated elements in tunicates are a case in point. Organic cocatalysts are chemical needs in some phylogenetic groups. The higher plants can synthesize all organic cocatalysts except vitamin B_{12}, whereas animals require nutritional sources of many of these. Microorganisms bridge the gap in biosynthetic abilities and are apparently the sole nutritional source of cobalamins. Porphyrins and sterols are required by many lower plants and animals, quinones and ascorbic acid are needs for a few higher animals, and vitamin A is needed by most higher animals. In each case, the evolution of the chemical need may be traced to the appearance of the specific biological function, the phylogenetic age of the requirement, and the availability of compounds which meet the requirement. There is ample evidence from the existing biochemical data to indicate that the present picture of requirements reflects but a minor portion of the total chemical needs of living organisms.

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CHAPTER 4

Biochemistry of Insect Metamorphosis

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- I. Introduction . . . 221
- II. Morphology and Physiology . . . 222
- III. Respiration and Respiratory Enzymes . . . 223
  - A. Cytochrome Oxidase . . . 224
  - B. Dehydrogenases . . . 225
  - C. Metabolism in Hemimetabola during Metamorphosis . . . 226
  - D. Conclusions . . . 226
- IV. Amino Acid and Protein Metabolism . . . 227
  - A. Free Amino Acids at Cocoon Spinning and Pupation . . . 227
  - B. Amino Acids at Puparium Formation of Diptera . . . 229
  - C. Peptides and Proteins . . . 229
  - D. Conclusions . . . 230
- V. Lipid and Carbohydrate Metabolism . . . 231
- VI. Phosphorus and Nucleic Acid Metabolism . . . 232
- VII. Special Metabolic Pathways Related to Metamorphosis . . . 232
  - A. Biosynthesis of Pigments . . . 233
  - B. Sclerotization . . . 234
  - C. Conclusions . . . 237
- VIII. Interaction of Metamorphosis Hormone with Genetic Material . . . 237
- IX. General Conclusions . . . 239
- References . . . 241

I. Introduction

Insect development is characterized by a series of progressive changes leading to the formation of the adult insect or imago (*1, 2*). The larva (or pronymph) emerges from the hatched egg. It differentiates to the imago directly in Hemimetabola and through an intermediate pupal stage in Holometabola. The transition from the fully grown larva to the adult organism is called *metamorphosis*. A great deal of successful work has been done on the physiology, especially the hormonal control, of metamorphosis. The neuroendocrine basis of this process has been demonstrated, and some of the active principles involved have been characterized.

In contrast to the physiology, the biochemical events which underly this significant phase of insect life remain to a great extent unknown. Many of the collected data are of a descriptive nature and give no insight into the inner mechanism of the transformation. They deal with quantitative and, less often, with qualitative variations of different metabolites during the different developmental periods. Respiration and basal metabolism have been amply investigated, while the study of intermediary metabolism has lagged far behind. A promising approach to the description of the successive steps of metamorphosis in terms of well-defined chemical processes is the elucidation of the biochemical mechanisms of hormone action. The isolation of active principles (one in crystalline form) has rendered such studies possible. An excellent review appeared recently (*2a*).

II. Morphology and Physiology

Owing to their rigid and impermeable exoskeleton, insects grow in successive steps. In order to increase its size the larva sheds its cuticle periodically. This process is called molting. Some insects attain the adult form gradually, showing progressive growth and differentiation; this is the case in the hemimetabolous insects. In the Holometabola (higher insects such as Diptera and Lepidoptera), there intervenes between larva and imago a period of drastic transformations—the pupal stage. It is characterized by a breakdown of larval tissues to an extent which differs from species to species, ranging from complete to partial tissue dissolution. An orderly sequence is retained, the last tissue to go being the fat body. Histolysis is followed by histogenesis of the imaginal structures from embryonic histoblasts, the less specialized and more plastic organs and cells being reconstructed *in situ* from larval elements.

In higher Diptera puparium formation often initiates the pupal stage. The imaginal development takes place in an environment isolated from the outside through the hardened insect cuticle. The chemistry of the sclerotization has been partly elucidated and is discussed elsewhere in this treatise by Pryor in Vol. IV, Chapter 8.

Diapause is a period of suspended animation and arrested growth with partial or total immobilization of the animal. It can occur in any of the stages of insect life and is often dependent on environmental stimuli.

Molting and metamorphosis are under hormonal control (see discussion in Chapter 5 of this volume). Three hormones are primarily involved in this process: (*a*) the brain hormone (secreted by the neurosecretory cells); (*b*) ecdysone, the hormone of the prothoracic glands; and (*c*) neotenin, the juvenile hormone produced in the corpora allata. Stimulation of the prothoracic gland by the brain hormone leads to secretion of ecdysone. Ecdysone induces all processes preparatory to the molt and also the molt proper. Simultaneous secretion of juvenile hormone leads to a larval molt. In the absence of juvenile hormone, development proceeds, resulting in the pupal and imaginal molt (Fig. 1).

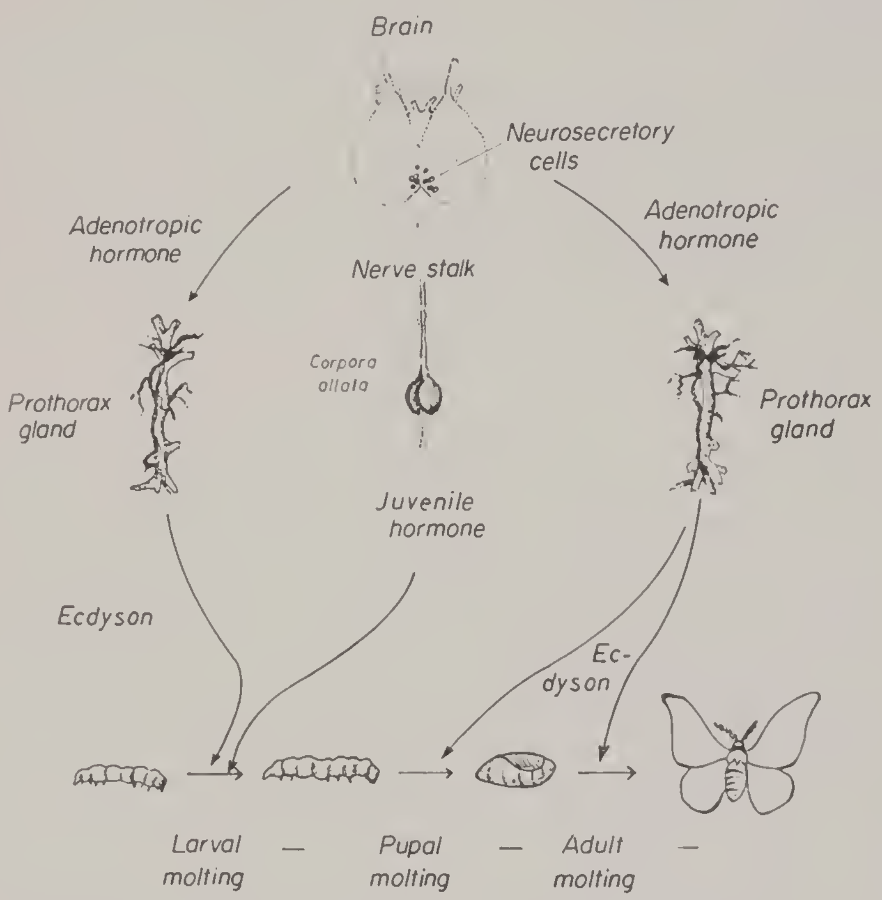

FIG. 1. Hormonal control of insect development. In the upper part, the hormonal glands and their secretions are shown. Below, the molts which are induced by the hormone(s) are shown. (From P. Karlson, "Introduction to Modern Biochemistry," English ed., Chapt. XX, p. 309. Academic Press, New York, 1963.)

Actually, all processes specific for metamorphosis are to be regarded as direct or indirect effects of the hormones, mainly ecdysone. This is most easily seen in animals deprived of their hormonal glands, which remain permanent larvae (*Dauerlarven*) or permanent pupae. However, the way in which these changes are brought about has not yet been elucidated, and the following paragraphs will be devoted principally to the biochemical phenomenology of insect metamorphosis.

III. Respiration and Respiratory Enzymes

Krogh (3), in 1914, first described the U-shaped respiration curve as being characteristic of metamorphosis. Since then respiration in various insect species has been studied and the results have confirmed the early findings of Krogh.

Agrell (*4, 5*) studied the aerobic and anaerobic utilization of energy during metamorphosis of the blowfly *Calliphora erythrocephala.* The respiratory activity of the pupa decreases during the first period of histolysis, then gradually increases during histogenesis and differentiation, forming a U-shaped curve. A similar course of oxygen uptake has been reported for *Musca* (*6*), for various lepidoptera [*Bombyx mori* (*7, 8*), *Hyalophora cecropia* (*9*)], and Coleoptera [*Popillia japonica* (*10–12*), *Tenebrio molitor* (*13–16*)]. In *H. cecropia* the lowest part of the respiration curve in normal pupae corresponds to the period during which development turns from histolysis to histogenesis (*9*). The respiration of pupae ligated shortly after pupation, which do not attain imaginal differentiation, shows a gradual decrease without the typical ascending part characteristic of histogenesis.

A. CYTOCHROME OXIDASE

The main enzyme involved in oxygen metabolism, cytochrome oxidase, shows the same type of curve in the course of pupal dormancy and development (*11, 17*). A most extensive study of the cytochrome system in *Hyalophora cecropia* during diapause and development has been made by Williams and associates (*18–30*). The rate of oxygen consumption follows the classic U curve, being high in larvae, falling during pupation to 1.4% of the mature larval value, then rising again a week prior to the termination of diapause. It was first suggested that this increase in metabolism is alone sufficient to explain the onset of imaginal differentiation. This could not be the case, however, since injury also causes an increase in metabolism to levels characteristic of postdiapausal insects, but does not initiate adult development.

The quantitative variations of the respiratory enzymes succinoxydase, cytochromes b, c, ($a + a_3$), and b_5 [first found by Sanborn and Williams (*19*) in the midgut of *H. cecropia*, but present in all tissues examined] were studied (*20–25*). Throughout metamorphosis all tissues, with the exception of the somatic muscles, show great variations in cytochrome concentrations. During diapause cytochromes b and c are not detectable, whereas in larval tissues they are found, together with cytochromes ($a + a_3$) and b_5, in moderate to abundant concentrations. Cytochrome ($a + a_3$) and b_5 concentrations decrease during diapause to low but still detectable values. With the initiation of imaginal differentiation (end of diapause) cytochromes b and c reappear and together with ($a + a_3$) and the microsomal cytochrome b_5 progressively rise in concentration.

The enzymes of the respiratory chain are located in the mitochondria.

Their decrease during diapause can be explained by mitochondrial destruction, which is in accord with the electron microscopic picture.

An interesting difference between respiration of diapausing pupae and insects in other stages of growth also was observed by Schneiderman and Williams (*26*). During active growth and metamorphosis the insects are sensitive to cyanide and CO, a phenomenon characteristic of the cytochrome oxidase. Diapausing pupae are insensitive and continue to live in presence of levels of cyanide and CO that normally are lethal.* The explanation for this phenomenon was sought in a terminal oxidase other than cytochrome oxidase. The identification of cytochrome b_5 in *H. cecropia* pupae was discussed under this aspect (*27*). Chefurka and Williams (*28*) noted changes in the prosthetic groups of flavoproteins with maximal concentration during diapause and decrease during adult development.

The explanation of this insensitivity was given by Harvey and Williams (*29, 30*) and Kurland and Schneiderman (*31*). Under normal oxygen tension the heart of diapausing *H. cecropia* pupae is cyanide and CO insensitive. Reduction of oxygen tension to low levels leads to a marked sensitivity of the heart to these respiratory poisons. There is a great excess of cytochrome oxidase in respect to cytochrome c, and this excess permits, under normal oxygen tensions, a certain amount of cytochrome oxidase to exist in active form, even in presence of CN^- or CO. This is enough for the low respiration of the diapausing pupa and gives the false impression of insensitivity of the pupae to these agents. The same results were obtained by Kurland and Schneiderman working with intact pupae. They also concluded that cytochrome oxidase is the terminal oxidase during all stages of life and that the observed insensitivity to CN^- and CO is due to the great excess of cytochrome oxidase over the other compounds of the respiratory chain.

B. DEHYDROGENASES

In a general survey of dehydrogenases in relation to histogenesis, Agrell (*4*) studied dehydrogenase activity in the head, thorax, and abdomen of *Calliphora* pupae. Total dehydrogenase activity shows a U-shaped curve similar to the respiration curve. It was shown that the rising part of this curve represents mainly an increased activity of dehydrogenases in the thorax. The thorax (and to a lesser extent the head) is the site of extensive histogenesis, muscle being formed from the dissolved fat body. Histogenesis (which also involves formation of enzyme proteins) is thus correlated with the ascending part of the U curve. On the other hand histolysis of the fat body (which can be shown histologically) accounts for the descending part of the curve.

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* This is a widespread characteristic of diapausing insects and was first detected by Bodine and Boell (*30a*) in diapausing grasshopper embryos.

Of the individual enzymes, succinic dehydrogenase has been the one most often studied. Its activity follows roughly the course of cytochrome oxidase and respiration; in *Musca* (32) and in *Tenebrio* it was especially low and may be the rate-limiting factor in the operation of the citric acid cycle. Some other dehydrogenases, namely α-glycerophosphate dehydrogenase, malic dehydrogenase (32) and alcohol dehydrogenase, also show the U-shaped variation in activity.

C. METABOLISM IN HEMIMETABOLA DURING METAMORPHOSIS

Zwicky and Wigglesworth (33) studied the rate of metabolism during the molting cycle of *Rhodnius prolixus* Stål (Hemiptera). The curves of oxygen consumption, CO_2 production, and heat generation show a series of depressions between molts. The part of the curve which extends from one ecdysis to the next can be considered, roughly, as a U curve comparable to the U curve of holometabolous insects. According to the authors, the curve of oxygen uptake is, primarily, a curve of protein synthesis and not of the amount of the respiratory enzymes present. The morphological transformations which lead to ecdysis, i.e., increase in cell constituents, formation of muscle fibrils and of cuticular proteins, are all connected with protein synthesis. This process, being endergonic, needs energy. The functional activity of the tissues, not the amount of respiratory enzymes, is the determining factor for oxygen consumption. This is very evident in *Rhodnius,* an insect in which high cytochrome stores in muscles persist from one stage to another while the respective respiratory curve in these periods greatly varies.

D. CONCLUSIONS

It is tempting to look at the changes in respiration as the controlling factor in development. Presumably the opposite is true: the needs of the developing tissues may control respiration. The main argument is injury metabolism. Diapausing pupae of *H. cecropia* when injured increase their respiration five- to tenfold, the rate being higher than in developing pupae. Yet they do not show any signs of differentiation; they just repair the wound and then go back to dormancy.

It is not clear how metabolism is stimulated in the case of injury. It has been proposed that subthreshold levels of ecdysone may be responsible; but injury metabolism occurs also in isolated abdomens, and it is more likely that some self-controlling mechanism adjusts oxidative metabolism according to need. This is also the argument of Wigglesworth, though experimental evidence is meager [see also the discussion in reference (*33a*)].

IV. Amino Acid and Protein Metabolism

One striking feature of insect hemolymph is the high content of free amino acids, first recognized by Florkin (*34, 35*) and regarded to be a taxonomic character for the insect group. There are considerable differences in the actual values for different species; the values range from 3 to 24 gm. per liter of hemolymph, as compared with to 0.02 to 0.9 gm. per liter of plasma for vertebrates. In periods of active development, especially during metamorphosis, great variations in the quantitative, sometimes also in the qualitative, content of amino acids are observed as a result of the histolytic-histogenetic phenomena concerned with imaginal differentiation (*36*). In the case of some amino acids, e.g.. tyrosine, fluctuation in their content reflects the onset of a specialized process in which these amino acids play a significant role.

A. Free Amino Acids at Cocoon Spinning and Pupation

Variations in amino acid content at different stages of development were studied by Wyatt *et al.* in *Bombyx mori* (*37*).

At the time of the pupal molt there is a temporary increase in the concentrations of alanine and glycine which then progressively fall again during pupal life. Glutamic acid increases during the pupal molt and stays at a high level in the pupa. Proline and lysine are found at higher concentrations in pupal than in larval life. The adult hemolymph shows a fall in lysine, glycine, and histidine concentrations, the main amino acids being glutamic acid and methionine. Amanieux (*38*) found an increased methionine content in pupae as compared to larvae.

Changes of amino acids during cocoon spinning have been studied by several authors (*39–43*). Florkin found a fall during spinning followed by a return to the former high level in pupae. Fukuda (*41*) compared the aminoacidemia of glandectomized silkworms with that of normal animals. Extirpation of the glands does not influence normal development. At the third day of the fifth instar the glandectomized animals showed slightly higher levels of proline, tyrosine, serine, threonine, and glycine; on the sixth day the difference was even greater. The amino acid concentration was restored at the time of the pupal molt. In glandectomized animals, Amanieux found a constant total amino acid content with greater concentrations of threonine and smaller of histidine than in the controls.

In the period between the last defecation and the pupal molt the concentration of glycine falls in normal animals. In glandectomized animals, there is an increase in concentration of glycine in comparison to the controls. During spinning serine concentration also falls, but during pupal life the amount of serine increases (*43*).

In *Hyalophora cecropia,* larval blood is characterized by the absence of free aspartic acid (*44*). Also, glutamic acid and glutamine are not detected in pupal blood. These amino acids appear only in the adult stage.

Hibernating pupae of *Sphinx ligustri* show an increase in glutamic acid and tyrosine at the end of diapause, but arginine, proline, histidine, alanine, lysine, and threonine progressively diminish (*43*). The diapausing pupae have a higher arginine, lysine, and proline content than the caterpillars, whereas the glycine content is higher in the latter (*45*). The same effect is to be seen in the diapausing pupae of *Smerinthus ocellatus* (*46*).

In the nondiapausing flour moth, *Ephestia kühniella,* the amount of ninhydrin-positive substances increases just after pupation and then progressively declines until the emergence of the insect. Ornithine, β-alanine, and phenylalanine are found only in the pupal stage (*47*). The fluctuation of tryptophan content in the (a+) and (a) strains were studied by Egelhaaf (*48*) in connection with biosynthesis of ommochromes. The concentration is small in larvae, reaches a maximum in the pupae, and then falls again.

Some observations in other insect orders may be mentioned here, which have some bearing on the histolysis-histogenesis problem. Del Vecchio (*49*) studied the distribution of the different nitrogen fractions (lipid soluble, water soluble, and water insoluble) during metamorphosis of the housefly *Musca domestica.* The results can be explained according to the general principal of tissue dissolution at the beginning of metamorphosis with respective increase of amino acids, and histogenesis in the latter part with corresponding amino acid decrease. In the Japanese beetle, *Popillia japonica,* Anderson (*50*) observed an increase in amino acid nitrogen at pupation followed by a gradual decrease during the remainder of the pupal period, while protein nitrogen changes in the opposite direction. This reflects tissue disintegration with a later histogenesis. Ludwig (*51–53*), on the other hand, found a lower amino acid content in the newly molted pupa at the time when Anderson believes that the greatest tissue destruction occurs. Considering the low amino acid content during pupation, Ludwig suggests that the end products of histolysis are removed as rapidly as they are added to the blood or that they are not discharged into the hemolymph.

B. Amino Acids at Puparium Formation of Diptera

Hackman (*54*) studied the amino acid composition of larval, prepupal, and pupal hemolymph of *Calliphora erythrocephala*. A qualitative difference was found only for hydroxyproline, which is present in larval and prepupal blood and is absent in the pupa. The total amino acid concentration falls from 6.6 mg. per milliliter of larval blood to 3.3 mg. in the prepupae, then rises again to 4.6 mg. per milliliter of early pupal blood. The greatest decrease occurs in the concentration of alanine, glutamic acid, glutamine, glycine, proline, and especially tyrosine. These changes reflect the biosynthetic processes that occur during cuticle formation and sclerotization. Glutamic acid may be used for the biosynthesis of glucosamine, the precursor of chitin. The cuticular proteins are rich in proline and tyrosine; these amino acids are thus used up in the formation of the cuticle. A large part of the tyrosine is metabolized to sclerotizing quinones which bring about hardening and darkening of the cuticle. This metabolic pathway has been recently elucidated by Sekeris and Karlson (*55*); it is discussed in Section VII,B.

Peptides and Proteins

It is obvious that histogenesis involves synthesis of proteins—structural proteins and functional proteins. On the basis of the observation that hemolymph peptides increased in the early pupal stage of *Calliphora erythrocephala*, Agrell (*5*) discussed the question whether peptides can be incorporated as units into proteins. Our present knowledge of protein biosynthesis seems to exclude this possibility. The increase of peptides in the hemolymph of *Calliphora* must be explained otherwise; perhaps they are incomplete breakdown products. The same may be true for the peptides 1 and 3 in *Ephestia* (*56*). It must be admitted, however, that our knowledge of the functional significance of the peptides in insect hemolymph is nearly nil.

Changes in blood proteins during larval life and metamorphosis have been reported for *Hyalophora cecropia* (*57*). With immunological methods, nine different proteins can be distinguished. Three are present in essentially constant concentration throughout larval and pupal life; three others appear in the last larval instar. The physiological significance of these proteins is not clear. According to Laufer (*58*), most proteins in insect hemolymph have enzymatic activity.

A correlation between protein changes and biochemical events of metamorphosis is not established. Only in the case of antigen 7 was a relationship found with a specific physiological process (*59*). It was shown that the protein exists only in female pupal blood in great concentrations. During the period of egg formation it is taken up by the yolk so that the concentration in the blood falls to one-twentieth of that in the yolk. It thus seems that the protein enters the ovaries and serves directly for the formation of definitive constituents of the future egg.

With the availability of C^{14}-labeled amino acids, studies on protein turnover became possible (*59a,b*). In *H. cecropia,* protein synthesis is possible also in diapause and is stimulated by injury; extra oxygen uptake in injured animals roughly parallels the protein turnover. The results are more relevant to the mechanism of diapause than to that of metamorphosis; with respect to the injury effect, it is interesting that a high rate of protein synthesis, even synthesis of cytochrome c, is possible without any visible sign of development. There must be a fundamental difference in the action of developmental stimuli—i.e., the hormones—and the effect of injury, though both involve the same metabolic machinery.

Recently, Bücher *et al.* (*60, 61*) studied the histogenesis of flight muscle in a hemimetabolous insect, *Locusta migratoria* L. About 3 days before the last (imaginal) molt, the muscles began to develop out of small filaments. During one phase, microsomes appeared; they are apparently formed in the nucleus and extruded through breakdown of the nuclear membrane. Mitochondria appeared somewhat later, as revealed by electron micrographs, and finally the enzymes of the glycolytic chain rose in concentration.

Similar observations on muscle development, mainly on the histological level, have been made by Wigglesworth (*61a*) in *Rhodnius.* In this case, some special muscles necessary for the act of molting developed and disappeared after the molt.

D. Conclusions

The numerous investigations on the fluctuations in amino acid content have not revealed points of special significance. The results are more or less those that are to be expected on the basis of histolysis, histogenesis, and protein biosynthesis. It must be kept in mind that the pupa is a closed system and that the amino acids needed in histogenesis have to be provided either by the pool of free amino acids or by breakdown of protein reserves. The latter occurs to a great extent: Even the proteins of the cuticle are digested before the molt by the molting fluid, which is rich in proteases, and the amino acids are reabsorbed (*62*).

The key problem in histogenesis seems to be the synthesis of structural proteins at the proper time and in the proper location. There are few studies on this problem, but it should be pointed out that insects may be well suited for these investigations, because they are—in the pupal stage—closed systems and the striking developmental processes are histologically well known.

V. Lipid and Carbohydrate Metabolism

Insects contain fairly large amounts of lipids (20–30% of their dry weight), which apparently serve as reserve substances. Changes in extractability during metamorphosis have been reported (*63*), but their significance is not clear. Agrell (*5*) found that about 50% of the energy metabolism is covered by fat utilization, carbohydrate being metabolized only to a small extent. The time course follows a curve roughly parallel to that for oxygen consumption.

It must be pointed out that some of the older calculations of fat utilization are based on determinations of respiratory quotients (R.Q.). Extremely low values, down to 0.2–0.3, were reported. It was later shown (*64–66*) that in several insect species the respiratory CO_2 is given off discontinuously, in discrete bursts. Only measurements over longer periods (8 hours or more) give reliable results, i.e., R.Q.'s of 0.8–0.9.

The major carbohydrate constituent of insect blood has been identified as the disaccharide trehalose (*37, 67, 68*). It has been found in all insects examined. Changes in trehalose concentration have been observed during metamorphosis in different species. In *Bombyx* the concentration falls from 250–600 mg per milliliter in fifth instar larvae to a minimum at pupation, then rises again during adult development (*69*). The same pattern of change is observed in the silkworm *H. cecropia* (*70, 71*), but not in *Deilephila euphorbiae* (*72*). Considering the increase in glycogen that occurs in many insects after cessation of feeding and the corresponding decrease in trehalose content during this period (*10, 70*) a conversion of trehalose to glycogen was suggested.

One metabolic feature of metamorphosis is the marked decrease in tissue glycogen content (*73–77*). The decrease in *Bombyx* at the end of the pupal stage reaches one-fourth to one-fifth of the initial value. Presumably glycogen is used by the metamorphosing insect as the source of energy. Corresponding to glycogen breakdown, a temporary increase in the lower sugars of the tissues is observed. Glycogenolysis is one of the few metabolic processes for which hormonal control has been experimentally verified. Ito examined the glycogen content of normal *Bombyx mori* pupae as well as in those ligated at the mesothorax within 3 hours after pupation (*74*). Glycogen content remains at a constant level in the operated animals, but it is lowered in normal pupa. This phenomenon is not a characteristic of metamorphosis, it occurs also in injury metabolism (G. Wyatt, personal communication).

Conclusions. The use of stored material for energy needs during metamorphosis is a consequence of the fact that no food is ingested in that period. The extent to which fat or rather carbohydrate reserves are accumulated and later used may vary with the species. So nothing specific to the biochemistry of morphogenesis can be derived from these studies.

VI. Phosphorus and Nucleic Acid Metabolism

Analyses of different phosphorus fractions are reported for *Antherea pernyi* (*78, 78a*), for *Bombyx* (*79*), and for *Calliphora* (*5*). There are fluctuations between inorganic phosphorus and various organic phosphorus fractions, but no conclusions can be drawn therefrom. Of more interest are studies of phosphorus exchange between different tissues. The midgut concentrates inorganic phosphorus, presumably by active transport. The exchange rate between tissues and blood is low in diapausing *H. cecropia* pupae and rises by a factor of 5 during the second day of development (*81*).

The nucleic acids (DNA and RNA) decrease during early pupal life and increase with progressing development (*51, 80, 82–84*). This is what would be expected on the basis of histolysis and histogenesis. Wyatt (*81*) studied the ratio of RNA:DNA during the early stages of adult development of the silkworm *H. cecropia*. The ratio increases during these early stages, a finding that can be attributed to increased RNA synthesis. The ratio then falls as development proceeds, presumably to increased DNA synthesis. Incorporation of P^{32} into RNA rises sharply early in adult development and later declines again.

Studies of the time course of P^{32} incorporation into wing tissue also led to the conclusion that RNA is synthesized at the onset of development. This fits very well with current ideas of the mode of action of ecdysone, i.e., activation of RNA synthesis at the site of the chromosome (see Section VIII). The DNA turnover observed later in development may reflect cell division during histogenesis.

Conclusions. Nucleic acid metabolism has been inadequately studied. From work on other animals, e.g., developing tadpoles (*85*), it would be expected that onset of development is characterized by a high nucleic acid turnover and eventually synthesis of some specific nucleic acids. More work along these lines could reveal the mechanism of development as controlled by gene activity.

VII. Special Metabolic Pathways Related to Metamorphosis

The metabolic changes during metamorphosis discussed so far are concerned with general aspects of metabolism: generation of free energy and its use for synthesis of proteins, nucleic acids, and other cell constituents. It has already been emphasized that they are believed to be controlled by hormones, since the whole process of metamorphosis is induced and controlled by hormonal stimuli. However, these changes are quantitative rather than qualitative, with the possible exception of synthesis of specific proteins. More insight into the biochemical mechanism of metamorphosis is to be expected from the study of specialized metabolic events characteristic for metamorphosis. Known examples are the synthesis of pigments (color change) and the sclerotization of the cuticle, which has been most intensely studied in the authors' laboratory.

A. BIOSYNTHESIS OF PIGMENTS

In some lepidopteran species, ommochromes are synthesized and deposited in the cuticle of the caterpillar shortly before cocoon spinning and pupation. In some cases this is correlated with changes in behavior and is of selectional value for the species (*86, 87*). In *Cerura vinula,* the process is controlled by the prothoracic gland hormone ecdysone. This has been concluded from ligation experiments and has been confirmed by direct injection of ecdysone.

This time course of color change is of some interest. Pigmentation of the epidermis occurs 6 days prior to pupation, at the time of mitoses in the wing anlagen. In ligated abdomens, the color change can be induced by ecdysone, doses of 50 Calliphora units (C.U.) being sufficient.* Injection of higher doses—up to 3000 C.U.—results not in color change, but immediately in molting (*88, 89*). The pupal cuticle laid down in this molt is rather thin; apparently, the epidermal cell has not had the time to accumulate and deposit sufficient cuticular material. It is concluded that the color change is an indicator that low concentrations of ecdysone are present in late larvae and that also in other species the secretion of ecdysone begins some time before the so-called critical period (i.e., the time when ligation can no longer prevent pupation). In *Cerura,* two critical periods are encountered, the one for prevention of color change being 8 days earlier than the one for pupation.

The pigments in the epidermis have been identified as xanthommatin and rhodommatin (*90*), whose structure is known through the work of Butenandt (*91*) and Butenandt and Schäfer (*92*). They are metabolites of tryptophan. Their biogenesis is known, but it has not yet been possible to detect the enzymes involved in their biosynthesis in insect material. It would be of interest to learn which enzyme is produced at the time of pigmentation.

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* See also Chapter 5, Section III,B, Table VI.

In *Locusta*, the color change (due to insectoverdin) is under the control of the corpora allata (*93*) and has no direct relation to metamorphosis (see also Chapter 5, Section III,B). In the papilionids (*Papilio xuthus* and related species) a pupal color variation has been observed which is related to the color of the environment at the time of pupation; this is controlled by neurosecretory centers in the brain and subesophageal ganglion (*94*).

B. SCLEROTIZATION

The hardening and darkening of insect cuticles has been termed sclerotization. It usually occurs shortly after the molt proper and gives rigidity to the exoskeleton.

There are many variations of the sclerotization in different orders and species. In flies, the maggots have a soft, unsclerotized cuticle which is transformed into the hard, brown puparium at the end of larval life. Pupation, i.e., the pupal molt, occurs later in the puparium. Puparium formation in the blowfly *Calliphora erythrocephala* has been the basis of the bioassay which has been used in the isolation of ecdysone from *Bombyx* pupae (cf. Chapter 5, Section III,B). Investigation of the biochemistry of puparium formation in order to elucidate the mode of action of ecdysone was therefore an obvious step.

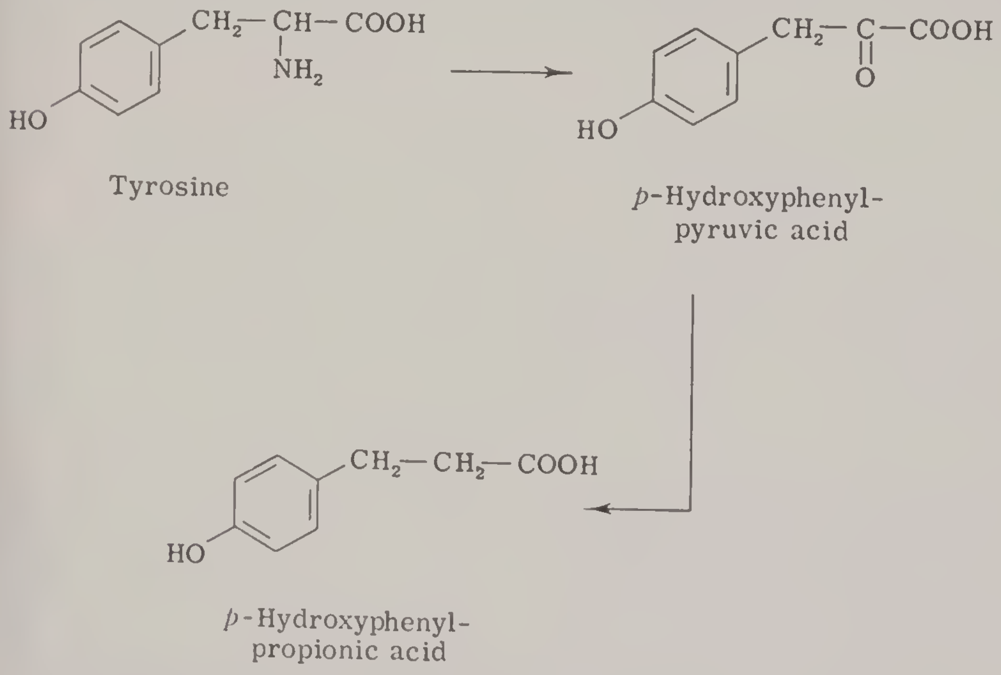
Some information on the chemistry of sclerotization was already available, mainly through the work of Pryor *et al.* (*95–97*). There are two main constituents in insect cuticle: a protein rich in tyrosine and proline, and chitin, a polymer of *N*-acetylglucosamine. The outer layer of the cuticle (epicuticle) contains lipids also and is rich in phenoloxidase, while the inner layer (procuticle) represents a network of protein and chitin fibrils. The hardening and tanning of the cuticle is brought about by the interaction of *o*-diphenols with the cuticular protein (*98*). The phenols are presumed to be oxidized in the epicuticle by the phenoloxidase to the corresponding quinones, which then diffuse into the procuticle. The reaction between the proteins and the "tanning agents," the quinones, will probably occur through the free amino groups of proteins, i.e., the ϵ-amino groups of lysine or arginine, and possibly with SH groups. Free NH_2 of chitin may also react. The result is a cross-linking of peptide chains, perhaps also of peptide and chitin molecules.

The assumption of quinone tanning has been substantiated through work with radioactive compounds (*99, 100*). When labeled tyrosine or dopa is injected into *Calliphora* larvae and the animals are allowed to pupate, the bulk of the radioactivity (75–80%) is found in the cuticle. The radioactive material present there is not tyrosine but some metabolite. Furthermore, the process is under the control of ecdysone; in ligated abdomens, it can be induced by injection of ecdysone preparations, but no incorporation occurs in the controls.

Other compounds have been tested as possible precursors of the quinones. Hydroquinone, proposed by Dennell (*101*) as a tanning agent, is not incorporated. *N*-Acetyltyramine, a substance first detected in insects (*102*), enters the cuticle to a considerable extent. The true precursor is *N*-acetyldopamine (see below).

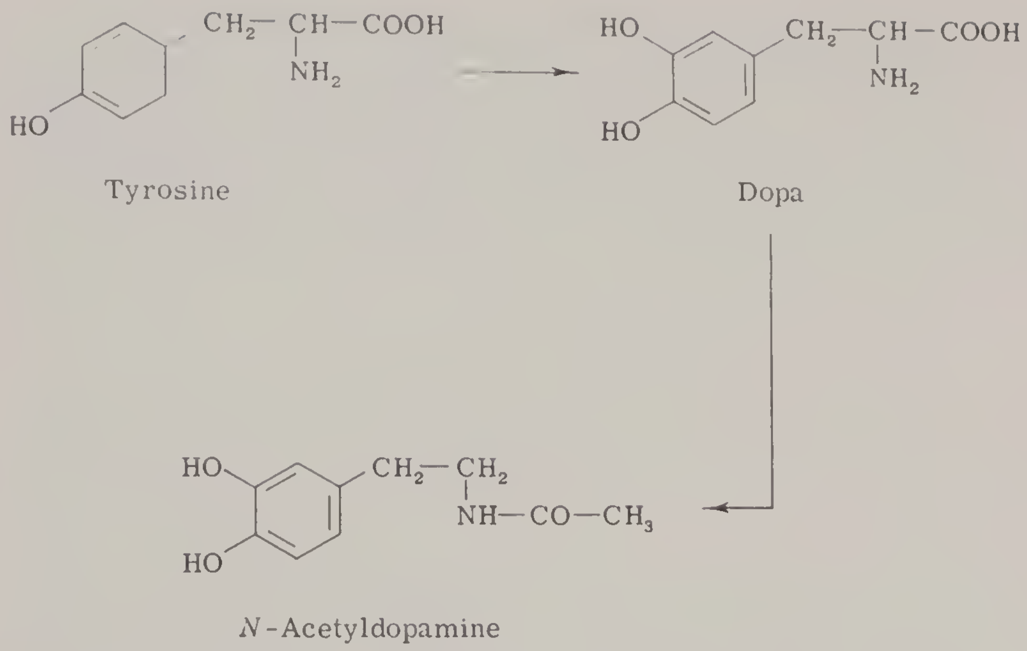
The main biochemical events leading to sclerotization are the biosynthesis of the diphenols and the activation of the pre-existing inactive form of the phenoloxidase. Both processes have been studied in *Calliphora,* and both have been shown to be dependent on hormonal action (*55, 103, 103a, 103b*).

In the early larval stages, tyrosine is metabolized through transamination to *p*-hydroxyphenylpyruvic acid (Reaction 1). This is accomplished by a soluble, pyridoxal phosphate-dependent transaminase and ketoglutarate as amino acceptor. The next metabolite is *p*-hydroxyphenylpropionic acid, which is presumably broken down in a way still unknown.



(1)

Toward the end of the third larval instar, the activity of the transaminase is greatly diminished. Instead, tyrosine is converted to dihydroxyphenylalanine (dopa), presumably by an TPNH-requiring enzyme system (Reaction 2). The so-called "tyrosinase" is not involved (see below). Dopa is decarboxylated and acetylated to yield *N*-acetyldopamine, a metabolite which is accumulated. This dihydroxy compound is a very good precursor of the cuticular quinones; injection of the labeled material leads to a high incorporation into the cuticle. Therefore *N*-acetyldopamine is believed to be the tanning agent of *Calliphora;* it is



(2)

accumulated some time before puparium formation and later enters the cuticle. The *N*-acetyldopamine which remains in the pupal body is transformed to the 4-*O*-β-glucoside, which exists throughout the pupal stage and is broken down to glucose and dopamine at the end of adult differentiation.

Oxidation of *N*-acetyldopamine is presumed to occur through phenoloxidases. Since the substrate of the phenoloxidase accumulates in late larvae and oxygen is also present, it was for a long time difficult to understand why they did not react. Work of Horowitz and Fling (*104*) gave the answer: The phenoloxidase system in *Drosophila* [and also in *Calliphora* (*103*)] is very complicated; it consists of a proenzyme, an activating enzyme, and eventually their product, the active phenoloxidase. The activation of the proenzyme follows autocatalytic kinetics.

The active phenoloxidase has been purified and crystallized, and its substrate specificity has been investigated (*105*). It is a true *o*-diphenoloxidase; it does not attack monophenols and has a marked specificity for dopamine, *N*-acetyldopamine, and dopa. Acid compounds (dihydroxyphenylpyruvic acid, protocatechuic acid) are not oxidized. The study of the enzyme, therefore, supports the conclusion that *N*-acetyldopamine is the sclerotizing agent.

The development of the phenoloxidase system in the third larval instar of *Calliphora* has been followed. Both components, the prophenoloxidase and the activating enzyme, rise in activity until puparium formation, then sharply decrease. These changes are also under the control of ecdysone: after destruction of the hormone-producing gland (Weismann's ring), the activity of both components remained constant for some time and then diminished. Injection of ecdysone into these animals produced a sharp rise, mainly of the activator enzyme (*103*). It is presumed that this is due to the *de novo* synthesis of enzyme protein.

C. Conclusions

The biochemistry of sclerotization is a good example of a metabolic mechanism restricted to metamorphosis and governed by the hormone of metamorphosis, ecdysone. The hormonal control is exerted at different points, the first being the shift in metabolic pathway of tyrosine metabolism. A new metabolic route is opened through biosynthesis of an enzyme protein (*103a*) and results in accumulation of the precursor of the tanning quinone. Simultaneously, the phenoloxidase system is built up first in the form of two components. At a certain time the whole process is set into motion; the brown puparium results within 1 to 2 hours. We do not yet know the final determinant of this process; the time needed from ecdysone injection to sclerotization is about 20–24 hours, visible changes and quinone incorporation occurring only in the last 2 hours. It is clear, however, that one of the main points of hormonal control in this case is on protein biosynthesis, i.e., enzyme production. The mechanism of this control will be discussed in Section VIII.

VIII. Interaction of Metamorphosis Hormone with Genetic Material

A new interpretation of hormone action in metamorphosis has become possible through the observation that ecdysone can act directly on the chromosomes, activating some genes and inactivating others. This observation was possible because Diptera have giant chromosomes in the salivary glands.

It is well known that the salivary gland chromosomes show a certain pattern of bands which have been identified as DNA. At certain times, some of these bands show the "puffing" phenomenon: the structure is loosened, the staining properties alter, and from radioautograms it is known that RNA is synthesized there (*106*). The puffing is generally interpreted as a sign of special activity of the genes (*107*).

The pattern of puffs changes during development. In *Chironomus tentans*, for example, a puff on the first chromosome (I R 19) disappears in the prepupae while a neighboring one (I R 18) arises from a small condensed band. Clever and Karlson (*108*, *109*) were able to show that this puffing on I R 18 can be induced by the injection of ecdysone. The response is very quick, the first signs being visible 30 minutes after injection. Within 2 hours 82% of the treated animals show the puff as compared with 5.9% of the controls. At the same time, the puff on I R 19 disappears in 30% of the treated larvae.

This result shows that ecdysone can induce changes in appearance, and presumably in activity, of the genetic material. Two points should be emphasized: the low dose which is needed [2×10^{-6} μg ecdysone (*109a*)] and the very quick response. It is by far the earliest response to ecdysone injection observed. Wigglesworth has seen changes in mitochondria (swelling) which occurred 6 hours after injection; the cuticular changes in *Calliphora* take over 20 hours to become visible. Therefore, one is tempted to conclude that the gene is the primary site of ecdysone action.

Actually, this idea has been put forward, without experimental evidence, by Schneiderman and Gilbert (*110*). They argued, mainly on theoretical grounds, that the nucleus would be a strategic place for hormonal action. The biological argument is that the whole program of development is genetically fixed. The genes contain the information on the nature of the events and processes to take place, the timing being under the control of hormones. It seems logical that the site of hormonal action is just the "information center," the gene.

There is another theoretical argument—a biochemical one. It has been shown above that in sclerotization as well as in cytochrome c increase, ecdysone induces synthesis of specific proteins, i.e., enzyme proteins. The pathway of protein biosynthesis has been elucidated in recent years (cf. Chapter 3 in Volume II of this treatise); it begins with the synthesis of a specific RNA (messenger RNA) which takes places under control or "priming" of DNA. The messenger RNA is then incorporated into the ribosomes which become active to synthesize protein. Precursors of the protein are the single amino acids which are activated by adenosine triphosphate (ATP), bound to a soluble RNA, and transferred to the ribosome where the peptide chains are formed. These mechanisms are shown diagramatically in Fig. 2 (*111*).

A hormone that stimulates protein biosynthesis could act on different points of this chain. A general increase in the level of ATP, for instance, could facilitate protein synthesis. However, if protein synthesis in general is not to be enhanced, but a specific protein is to be synthesized, stimulation must occur at a point where specificity of "information" is handled. This is either the messenger RNA or the site of its production, the gene.

Conclusion. It may be premature to draw conclusions from this work: it has developed only recently and its generalizations are not easy to evaluate. Much more work is necessary to confirm the hypothesis stated above and to fill the general framework with facts. On the other hand, the observation that ecdysone induces puffing opens a new line of attack, and more refined experiments can be devised to prove the hypothesis.

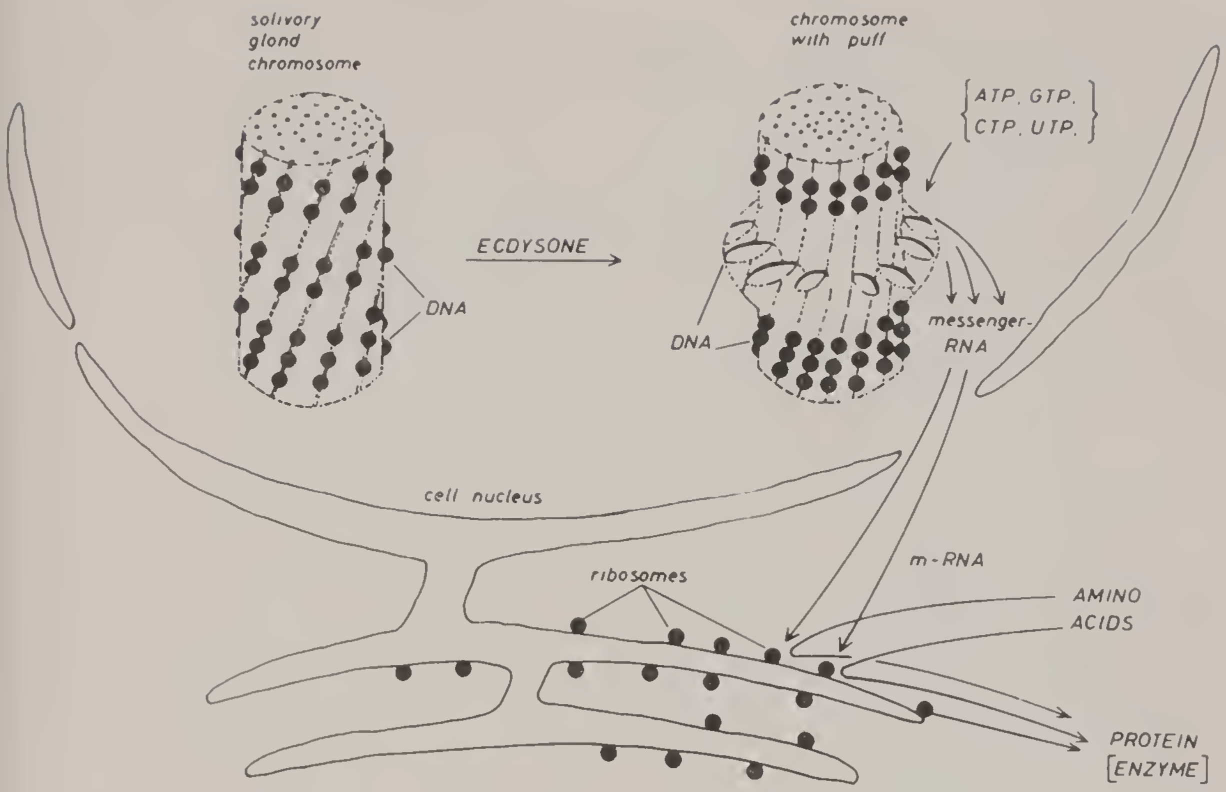


FIG. 2. Schematic representation of the interaction of ecdysone with genes. Through the action of ecdysone, a certain site of the chromosome is activated to produce RNA from precursors. The RNA attaches to the ribosomes where it serves as template for the synthesis of a specific protein, e.g., an enzyme. This chain of events may also apply to vertebrate hormones, especially those which stimulate biosynthesis of specific proteins. [From P. Karlson, *Perspectives in Biol. Med.* 6, 203 (1963).]

Furthermore, a number of current ideas on developmental processes as well as present knowledge on genetic information and protein biosynthesis fit well with the conclusion that the primary site of action of ecdysone is the gene, which then produces specific RNA, thus initiating synthesis of specific proteins. This may be more than coincidental.

IX. General Conclusions

The biochemistry of insect metamorphosis has been studied from three different aspects. The first one is descriptive—the study of fluctuations of metabolites and activity of enzymes in the course of metamorphosis. The second is physiological—involving the hormones which govern the whole process and the details of hormonal control. The third is a combination of the first two. Its working hypothesis is that the biochemical changes are brought about by hormones. This leads to a study of the mode of action of the hormones. It is clear that this approach is possible only if there is some knowledge of the biochemical process to be induced as well as of the hormones involved.

At the present time, some basic facts are known on the first and the second levels. Changes in respiration and in activity of the respiratory enzymes, breakdown and synthesis of proteins, and special processes like sclerotization have been studied and correlated with the hormonal action. Also, the hormones involved have been obtained in form of active extracts, in case of ecdysone even crystalline. With regard to the third question, the mode of action of the metamorphosis hormone(s), there are at least some new ideas which arose from an unexpected finding—the interaction of ecdysone with the genetic material. *A priori* one would expect rather the opposite effect. The whole development of any animal —vertebrate as well as insect—is, genetically fixed. This holds for main events like voltinism, number of instars, etc., as well as for minor morphological characters like bristles or wing patterns. If the development, then, is governed by genes, one would expect some genic activity to induce the release of the hormone, i.e., the brain hormone which then stimulates the prothoracic gland.

Our experiments have by no means excluded this possibility. They have only demonstrated that the hormone ecdysone acts on some genes —those which normally become active in the prepupal period—and produce the puffing. This phenomenon is generally believed to indicate gene activity, RNA synthesis, etc. But this leads to the idea that the activation of a gene would set into motion a chain of events which give rise to protein synthesis, perhaps enzyme synthesis. This mechanism can obviously shift metabolic pathways and by interaction with other determinants can account for a number of developmental processes. One might say that pieces of stored information are released and put to work. This is, however, a framework of ideas which is to be filled by exact knowledge of the single steps. In some cases, e.g., enzymes involved in tyrosine metabolism, glycogen breakdown, it has been possible to demonstrate the relation between the hormone and the rise in enzyme concentration. In other cases, e.g., cytochrome c production, this could be brought about not only by the hormones, but also by injuries, so that the real cause is not yet clear.

Insect metamorphosis is a good example of morphogenesis. Its study is in several respects easier than that of embryogenesis. The aim of this study is to understand in a scientific, i.e., a causal, way the chain of events leading from the larva to the fully grown insect, the adult. Much remains to be done to arrive at this goal, but some lines can already be drawn, as we have tried to do in this review.

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CHAPTER 5

Hormones in Invertebrates*

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I. Introduction 246
II. Classification 247
III. Neurohumors 248
  A. Acetylcholine 248
  B. Adrenaline and Noradrenaline 253
  C. 5-Hydroxytryptamine 255
IV. Products of Neurosecretion 256
  A. Flatworms 257
  B. Polychete Annelids 258
  C. Oligochetes 259
  D. Hirudinea 260
  E. Sipunculoidea 260
  F. Mollusca 260
  G. Arthropoda 261
V. Hormones Elaborated by Tissues Other Than the Nervous System . . 270
  A. Mollusks 270
  B. Arthropods 271
  C. Protochordata 282
VI. Pheromones 285
  A. Sex Attractants 286
  B. Pheromones of Social Insects 287
VII. Concluding Remarks 288
  References 290

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* Parts of this chapter have been translated from the French by Dr. Erich Hirschberg, College of Physicians and Surgeons, Columbia University, New York.

I. Introduction

The preparation of a chapter devoted to the comparative biochemistry of hormones among the invertebrates is fraught with considerable difficulties, above all because of the fragmentary nature of the available evidence. Actually, the existence of humoral correlations has been demonstrated in various invertebrates by observation and experimentation but the intervention of specific substances, which may be defined as hormones in the strict sense of the word, has not always been shown with certainty. Most invertebrates are much less accessible to experimentation than the vertebrates commonly used in the laboratory, and even the definition of the term "hormone" in the invertebrates is still characterized by a certain lack of precision. Frequently, actions which are tentatively considered "hormonal" may correspond to general metabolic effects and additional investigation may be required before conclusions can be drawn.

The scarcity of facts, which is already evident when the physiological aspects of the problems of invertebrate endocrinology are considered, becomes even more apparent when attention turns to biochemical aspects. Actually, only certain hormones of insects have been studied with the techniques of chemical analysis; at this time, a survey of structural biochemistry devoted to invertebrate hormones can encompass only a single class of the phylum Arthropoda. Apart from precise data concerning the Insecta and the Tunicata, and some indications about certain hormones in the Crustacea, the only information strictly in the area of analytical biochemistry bears on the solubility characteristics of presumed hormonal principles.

The situation is even more unfavorable when the question is considered with respect to comparative biochemistry: in fact, even the findings established with certainty in insects and crustaceans are based on the examination of a very small number of species, and this makes even the slightest attempt at comparison a most difficult undertaking. The morphological, physiological, and ethological differences among representatives of the same phylum are generally greater for the invertebrates than for the vertebrates, so the generalizations which would be permissible for the latter cannot be made for the former. The functional significance of certain organs and their homology from one class to another are often imprecise among the invertebrates, so that it is not possible to study their secretions within the framework of the biochemistry of evolution. A genuine comparative investigation of the hormones of invertebrates would appear quite premature on the physiological, and *a fortiori* on the biochemical, level. In the absence of this synthesis, which at the present stage of our knowledge is not attainable, the objective of this chapter is to pose problems rather than resolve them, to bring together the few indications that may orient biochemical research toward physiological problems which are of great interest but most difficult, and frequently impractical, to approach.

II. Classification

The paucity of data mentioned above underlines the impossibility of classifying the hormones of invertebrates according to their chemical constitution. In the absence of such a classification, which appears to be reserved for the very distant future, the site of elaboration of the hormonal principles and their modes of action provide the elements which permit four groups to be recognized.

Certain principles elaborated by nerve cells are produced in the immediate vicinity of the receptor organ; they travel a very short distance and act during a brief period. These principles constitute the group of the *chemical intermediaries of nerve transmission, or neurohumors* (*1*); acetylcholine, 5-hydroxytryptamine, noradrenaline, and adrenaline are examples.

Other hormonal principles, likewise elaborated by nerve cells, are subjected to transport over a very large distance and to storage after being liberated. They act for a longer time than the neurohumors and play a role as coordinators of neuroendocrine function. These compounds are called *products of neurosecretion* (*1–3*). It is appropriate to mention here a disadvantage of this nomenclature, that is, the risk of confusion with the product of secretion which, in the perikaryons that elaborate these principles, can be demonstrated by histological techniques. However, this stainable product is in all probability merely a carrier of the active principles; thus the same term is used by the morphologist and the physiologist with very different meanings. In the vertebrates, this second category is represented by the so-called posterior pituitary hormones; in the invertebrates, the principles elaborated by the protocephalic neurosecretory cells of the Arthropoda are the best-known example. Recently certain authors have proposed the collective name of neurohormones or neuroendocrine substances for the materials corresponding to the first two categories.

A third group of hormonal principles elaborated by the organism of invertebrates consists of *substances secreted by tissues other than the nervous system*. Ecdysone of insects, the compound of the same type elaborated by the molting gland of the malacostracan crustaceans, and the juvenile hormone of the insects are examples of this category.

The fourth and last group is composed of substances which are active at very low doses, are produced into the external medium—in contrast to genuine hormones—and act from individual to individual. These substances, which have been found only among the insects, have been designated as *pheromones*.

Our knowledge about the neurohumors can be detailed in an organized fashion. The same cannot be said about the information available on the products of neurosecretion and on the glandular hormones proper, for which we have deemed it necessary to review the fragmentary notions now at hand according to the zoological order. Section VI, devoted to the pheromones, deals solely with results obtained in insects. Only the acquisition of further knowledge, which will occur at an as yet unforeseeable time, will make it possible to relate the hormonal activities studied here to defined chemical structures rather than to their distribution in classes of animals.

III. Neurohumors

In essence, the group of neurohumors is represented by a quaternary ammonium base, acetylcholine, two catechol amines, noradrenaline and adrenaline, and an indole amine, 5-hydroxytryptamine. Other compounds, related to those listed, have been reported in various invertebrates. Thus, the hypobranchial gland of the gastropods of the genus *Murex* contains urocanylcholine (*4*) and certain tissues of insects contain dopamine (hydroxytyramine); the role of these compounds in the organism of the invertebrates in which they occur, however, is unknown at present, and they cannot be considered as neurohumors.

A. ACETYLCHOLINE

Among all the neurohumors, acetylcholine has been studied most intensively and is most widely distributed. Table I, based on the results of Bacq (*5*), shows that this compound is absent from the sponges and coelenterates, but that it is found in the listed representatives of other invertebrate phyla with the exception of the tunicates. It has been detected in some protozoans.

It is appropriate to emphasize, with Bacq (*5*), that the presence of acetylcholine in the tissues of an animal does not by any means prove the existence of cholinergic mechanisms of nerve transmission. Actually, the notions which have been clearly established on the subject of the physiology of the cholinergic nerves of the vertebrates lead to the formulation of the following three requirements when a presumptive cholinergic system is studied in an invertebrate: (*a*) sensitivity of the receptor tissue to very small concentrations of acetylcholine; (*b*) the existence, in a biologically inactive form, of a reserve of acetylcholine

TABLE I

Existence of Cholinergic Mechanisms in Invertebrates[a]

Animal group	Presence of acetylcholine	Presence of cholinesterase	Sensitivity to acetylcholine	Action of eserine	Action of atropine and curare
Porifera	0	0	?	?	?
Coelenterata	0	0	0	0	0
Annelida	+	+	++++	++++	++
Crustacea	+	+	0 or +	0	?
Arachnida	?	+	?	?	?
Insecta	?	+	?	?	?
Mollusca	++	+	+ to ++++	0 to ++	0 to ++
Echinodermata	+	+	++++	++++	++
Tunicata	0	+	+or 0	0	?

[a] According to Bacq (*5*).

which can be extracted by trichloroacetic acid or acid alcohol; (*c*) the existence of cholinesterase.

Indeed, acetylcholine is distributed very widely, in the plant kingdom as well as in all animal tissues. The classic example of the mammalian placenta shows that the biochemical equipment required for cholinergic functioning may exist without being utilized; in fact, the placenta, an organ devoid of nerves, is rich in acetylcholine and contains cholinesterase. Experimental proof of the cholinergic functioning of nerve transmission is provided by the increase and prolongation of the effects of nerve stimulation in the presence of eserine and by the diffusion into the medium, during this stimulation in the presence of a cholinesterase inhibitor, of a substance endowed with the pharmacodynamic properties and chemical characteristics of acetylcholine.

1. *Distribution in the Animal Kingdom*

Certain Infusoria of the genus *Paramecium* contain acetylcholine, which obviously does not have neurohumoral significance here.

In the sponges, there is neither acetylcholine nor cholinesterase (*6*).

Acetylcholine has been demonstrated in planaria (*7*); it is found in representatives of all the subdivisions of the old heterogeneous group of the worms. In annelids and sipunculids, the compound is localized primarily in the muscles. Moreover, cholinesterase has been shown to be present in these animals (*6, 8, 9*), and experiments prove (*10*) that the motor nerves in the annelids and sipunculids are cholinergic like those of the vertebrates.

The digestive tube and the longitudinal muscles of the holothurians

(Echinodermata) are rich in acetylcholine (*6, 8*); cholinesterase is present and the sensitivity of echinoderm muscles to acetylcholine has been demonstrated experimentally.

The distribution of acetylcholine in the tissues of Arthropoda and the existence of cholinergic mechanisms among representatives of this phylum have given rise to numerous discussions.

Actually, acetylcholine is present often in appreciable amounts, in the nervous tissue of crustaceans (*8, 11, 12*), whereas no cholinesterase is found (*11, 13, 14*) and the muscles of the same animals are devoid of acetylcholine (*6, 8, 9, 12, 15*). The hemolymph of arthropods has only very slight cholinesterase activity. Some authors therefore conclude that nerve function in arthropods does not involve cholinergic mechanisms and recall on this point the very low sensitivity of the muscles and nerves of these animals to relatively high doses of acetylcholine. Other investigators believe that cholinergic mechanisms may exist.

Among the mollusks, nervous tissue is very rich in acetylcholine and acetylcholinesterase activity is pronounced (*6, 8*). Circulating blood is rich in cholinesterase but does not contain acetylcholine. The acetylcholine content of the myocardium is low in the cephalopods (*16*), the lamellibranches, and certain gastropods, but high in other gastropods (*16*). With regard to the sensitivity of the acetylcholine receptors, Table II, reproduced from Bacq (*5*), shows that, of all materials studied, the heart of *Venus mercenaria* is by far the most sensitive. The reaction of this tissue is one of inhibition; but the inhibition caused by electrical excitation of the visceral ganglion is intensified and prolonged by eserine (*17*). In this way, the cholinergic character of the cardiac restraint is demonstrated in a representative of the class of lamellibranches: the manner of reaction during excitation of the inhibitory nerve, more-

TABLE II

SENSITIVITY OF VARIOUS ORGANS OF MOLLUSKS TO ACETYLCHOLINE[a]

Species	Organ	Active concentration
Octopus vulgaris	Median ventricle	2×10^{-6}
	Tunic	1×10^{-6}
Eledone moschata	Tunic	1×10^{-4}
Helix pomatia	Ventricle	1×10^{-6} to 1×10^{-5}
Ostrea edulis	Ventricle	1×10^{-5}
Buccinum undatum	Foot	1×10^{-5}
Aplysia punctata	Foot	1×10^{-5}
Venus mercenaria	Heart	1×10^{-12} to 1×10^{-9}

[a] According to Bacq (*5*).

over, is reminiscent of what is seen in a frog heart during excitation of the vagus nerve.

With respect to cholinergic mechanisms, the tunicates, animals near the vertebrates in zoological classification, are quite different from them. The mode of neuromuscular transmission of the arthropods and the coelenterates appears to diverge widely from that of the vertebrates. Indisputable examples of cholinergic innervation exist among the mollusks, and the heart of *Venus* has proved the object of choice for the physiological and biochemical study of acetylcholine. As pointed out by Bacq (5), the chemical mechanism of neuromuscular transmission of the annelids and echinoderms seems to approach, in many ways, what is known on this subject among the vertebrates.

2. *Biosynthesis, Accumulation, Axonal Transport*

The biosynthesis of acetylcholine has given rise to a large number of investigations (cf. references *18–23* for the pertinent bibliography) bearing on the tissues of vertebrates; to recall these, even in summary form, would be out of place here. It is generally agreed that every part of the nerve cell can be the site of this synthesis, but assays in nervous tissue show that its concentration is higher in areas containing many perikaryons; therefore, synthesis would be more active in these cells than in the dendrites and the axons.

Direct proof is still lacking for transport of acetylcholine by way of the axons, but this route appears probable.

The accumulation of acetylcholine in the regions rich in nerve endings has been demonstrated by assay of this compound. Histochemical techniques do not permit the demonstration of acetylcholine and therefore provide no information with regard to the localization of this compound in the synapses. Electron microscopy shows, in the presynaptic endings of various neurons, vesicles with a diameter of 20–50 mμ; a distinct membrane surrounds these synaptic vesicles, which may be the site of the chemical intermediaries of nerve transmission. Particularly clear pictures of the synaptic vesicles are seen in the myoneurol synapses of the hymenopteron *Vespula carolina* (*24*).

3. *Site and Mode of Action*

As pointed out by Welsh (*2*), acetylcholine is very widely distributed in animal tissues and all cells are sensitive, to some degree, to this compound; it is also true that in certain cells the sensitivity to acetylcholine is well localized. Thus, the regions of the striated muscle fiber where the myoneural synapses are located are significantly more sensitive than the rest of the fiber. Likewise, the perikaryon of a sympathetic ganglion react to an application of acetylcholine which would be without effect if made at the level of the axons. This fact may be the reason for the insensitivity of arthropod muscles to acetylcholine, which contrasts with the presence of an appreciable quantity of this compound in nervous tissue. It is possible, that acetylcholine which may be liberated at the level of the nerve endings can reach the "receptors" whereas the same compound applied by the investigator to the surface of the muscle, cannot. These "acetylcholine-receptive" compounds are probably grouped near the cell membrane, as the rapidity of action of acetylcholine suggests; however, they have never been directly demonstrated.

In the absence of precise knowledge about the structure of the "receptor" substances, recent investigations have provided data on the relations between the chemical structure of acetylcholine and its pharmacodynamic effects. Some of the most clearly defined among these results have been obtained on the heart of *Venus mercenaria* (*25–28*). The importance of the position occupied by the cationic group in the molecule is as pronounced in the heart of *V. mercenaria* as in other test objects; tetramethylammonium ions act on the heart of *V. mercenaria* in the same way as acetylcholine, but the depressant action is much weaker. The triethyl analog of acetylcholine and the tetraethylammonium ions, on the contrary, have an inverse action; they excite the heart of *V. mercenaria,* and this may be due to a block of endogenous acetylcholine. The progressive substitution of ethyl groups for the methyl groups of tetramethylammonium ions shows that the depressant action exerted on the heart of Venus is maintained entirely when the compound has three CH_3 groups; it decreases significantly when the number of methyl groups drops to two; and it disappears completely when one of these is replaced by another alkyl group.

The length of the carbon chain also plays an important role. A systematic study of the effects exerted by different compounds of the *n*-alkyltrimethylammonium series shows that maximal cardiac restraint is produced by *n*-amyltrimethylammonium whose 5-carbon chain approaches, in length, that of acetylcholine (*25, 26*).

However, the activity of this compound is one-seventieth that of acetylcholine, a fact which of necessity leads to a search for the presumed role of the carbonyl group of the acetylcholine molecule. Experiments show (*27*) that 4-ketoamyltrimethylammonium is significantly more active than the compounds carrying the carbonyl group in the 3- or 2-position; it is important to point out that the variations in activity according to the position of the carbonyl groups are the same for the isolated heart of *V. mercenaria* and for a whole series of classic pharmacodynamic tests of acetylcholine (Table III).

TABLE III
MOLAR CONCENTRATIONS WITH EQUAL ACTIVITY OF SOME ANALOGS OF ACETYLCHOLINE[a]

Compound	Rabbit ear	Guinea pig ileum	Frog heart	Frog rectum	*Venus mercenaria* heart
Acetylcholine iodide	1	1	1	1	1
4-Ketoamyltrimethylammonium iodide	50	80	416	1.1	12
3-Ketoamyltrimethylammonium iodide	250	670	1670	1.3	34
2-Ketoamyltrimethylammonium iodide	1660	330	5010	153	620
n-Amyltrimethylammonium iodide	44	8	325	13	70

[a] According to Welsh (*2*).

These results agree with the hypothesis of a chemical binding between acetylcholine and the receptor compound; most probably, the cationic nitrogen of the molecule unites with an anionic group of the receptor molecule. This union does not seem feasible when the nitrogen atom is surrounded by groups which are larger than the methyl group. Moreover, it is probable that the carbonyl group of acetylcholine combines with the "receptor" compound.

Further, the reactivity of the heart of *V. mercenaria* to acetylcholine in the presence of various blocking agents demonstrates that the receptor molecule probably has properties intermediate between those of receptors present in the vegetative ganglia of the vertebrates and those in their striated muscles.

On the whole, those fundamental biochemical mechanisms of the cholinergic systems which have been studied well in this regard exhibit common characteristics whether vertebrates or invertebrates are involved.

4. *Tropic Action of Acetylcholine*

Apart from its participation in the chemical transmission of nerve impulses, acetylcholine—which is present in many tissues that have no direct relation with the nervous system—may play a role in cellular metabolism. A tropic action of this compound had been established in vertebrates (*29*) and in *Planaria* (*2*).

B. ADRENALINE AND NORADRENALINE

Our knowledge about the sympathomimetic catechol amines in invertebrates is most fragmentary.

The presence of adrenaline (epinephrine) in annelids has been accepted since the discovery of pheochrome cells in the ganglia of the ventral nerve chord in *Hirudo medicinalis* (*30, 31*); extraction by classic techniques of the nerve ganglia of this leech yields a significant amount of adrenaline which has all the pharmacodynamic properties of the levorotatory adrenaline of the vertebrates (*5*).

Investigations on adrenaline in insects have given rise to contradictory conclusions. In fact, Wense (*32*) obtained significant quantities of adrenaline from the larvae of the coleopteran *Tenebrio molitor* whereas Gregerman and Wald (*33*) showed with chromatographic techniques that the body of the larva of *Tenebrio molitor* does not contain adrenaline but rather two other *ortho*-diphenols. More recently, adrenaline, noradrenaline, and hydroxytyramine have been identified in the extracts of *Tenebrio molitor* and *Apis mellifera* (*34, 35*).

The steps in the biosynthesis of the sympathomimetic catechol amines are now known, but only the tissues of vertebrates have been studied in this respect. Accumulation of these catechol amines at the nerve endings is considered probable, but precise data are lacking on the form in which they are stored in the adrenergic nerves. We shall not consider here the problems bearing on the accumulation of adrenaline and noradrenaline in the vertebrate adrenal tissue.

The mode of action of adrenaline and noradrenaline has given rise to the same hypotheses as those mentioned above with regard to acetyl-

TABLE IV

SOME EFFECTS OF ADRENALINE IN INVERTEBRATES

Organ	Animal group or species	Effect	Reference
Heart	Vertebrata	Increased frequency and amplitude	Many
	Cephalopoda	Increased frequency and amplitude	(*37, 38*)
	Crustacea	Increased frequency and amplitude	(*39, 40, 41*)
	Limulus	Increased frequency and amplitude	(*42*)
	Leptodora	Depression	(*43*)
	Ostrea	Stimulation (small doses) Inhibition (large doses)	(*44*)
	Anodonta	Inhibition (large doses)	(*45*)
	Helix	Inhibition	(*46*)
	Pecten	Excitation	(*39*)
Denervated muscle	Annelida	No effect	(*39, 47*)
Innervated muscle	Annelida	Excitation	(*39, 47*)
Muscle	*Holothuria*	Excitation	(*48*)
Cloaca	*Holothuria*	Inhibition	(*49*)
Striated muscle	Crustacea	Excitation	(*50*)

choline. It is very probable that these compounds are involved in the immediate vicinity of the cell membranes; studies devoted exclusively to the organs of vertebrates suggest the presence of "receptors" as in the case of acetylcholine, as well as the existence of definite relationships between the chemical structure and the reactivity of the compounds with the cellular receptors. The classic inversion of the effects of adrenaline, well known in vertebrate organs, occurs also in the retractor muscle of the byssus of *Mytilus edulis* (*36*).

It is appropriate to mention that the pharmacodynamic data on the action of adrenaline on the heart, the smooth musculature, and the metabolism of the invertebrates are quite contradictory (Table IV); adrenergic mechanisms of nerve transmission probably exist in the annelids, but the effects on the heart and smooth musculature of many invertebrates do not appear to be specific.

C. 5-Hydroxytryptamine

This indole amine has been demonstrated in a large number of tissues in various animals; its distribution is such that its classification among the neurohumors has only quite recently been accepted. In fact, 5-hydroxytryptamine is found in the digestive tract of all vertebrates, in mammalian blood platelets, and in some animal venoms.

Among the invertebrates, 5-hydroxytryptamine has been detected in the digestive tract of the tunicates. The mollusks contain appreciable amounts; nerve centers in *Busycon canaliculatum*, *Venus mercenaria*, and *Sepia officinalis* are rich sources. Moreover, the posterior salivary glands of *Cephalopodes octopodes* contain considerable quantities, as does the median zone of the hypobranchial gland in *Murex trunculus* (*51–53*).

The nervous tissue of the crustaceans contains quantities of 5-hydroxytryptamine of the same order as those found in the nerve ganglia of mollusks (*52*). In addition, the venom of *Hymenoptera* and that of the scorpions represent localizations of this indole amine in the arthropods.

Among the coelenterates, *Physalia* and the sea anemones contain 5-hydroxytryptamine, the nematocysts representing the main localization (*51*).

One of the principal pharmacological properties of 5-hydroxytryptamine, namely its ability to cause contracture of the smooth musculature, was discovered in mollusks and coelenterates. Thus, the heart of *Venus mercenaria*, that of *Buccinum*, the sphincter of sea anemones, the retractor muscle of the byssus of mussels, and the heart of *Anodonta* have been proposed as test objects for the biological assay of serotonin.

Precise data are not available concerning the site of elaboration and accumulation of 5-hydroxytryptamine in the nervous tissue of the invertebrates. The production of this compound by other tissues, notably by the posterior salivary gland of octopod cephalopods and by the hypobranchial gland of *Murex trunculus,* has been related to the presence, in the cells of these organs, of characteristic secretory glands (*53*).

The inclusion of 5-hydroxytryptamine in the group of neurohumors is based primarily on experiments with mollusks. In fact, it considerably augments the rhythm and amplitude of cardiac contractions in lamellibranches and gastropods (*54*); these effects are very close to those of electrical excitation of cardioacceleratory nerves (*54*). Further, it determines the relaxation of the anterior retractor muscle of the byssus of *Mytilus* (*36*) and may represent the mediator of the inhibitory nerves of this muscle. Certain observations suggest that this may be true also of the crustaceans (*52*).

The stages in the biosynthesis of 5-hydroxytryptamine, by oxidation of tryptophan and decarboxylation of 5-hydroxytryptophan, are well understood primarily as a result of studies carried out in mammals and bacteria; several pathways of degradation in man and other mammals are known, but the biochemical aspects of the metabolism of 5-hydroxytryptamine in the invertebrates have been described only very incompletely.

IV. Products of Neurosecretion

In a general way, the available data on the endocrine correlations in the vertebrates on the one hand, and in the invertebrates on the other, differ significantly by the place occupied by the phenomena of neurosecretion in the two types of organisms. In effect, the elaboration of hormonal principles by certain hypothalamic nuclei plays an important role in the endocrine equilibrium of the vertebrates, but the amount of information acquired about other endocrine glands is considerable. In the invertebrates, it is the phenomena of neurosecretion that constitute the best-understood part of the endocrine correlations and only a very few endocrine glands entirely unrelated to the nervous tissue have been studied well. This state of affairs explains the emphasis on neurosecretion in the preceding paragraphs; it also throws light on the difficulties inherent in the biochemical study of its products. At the present time, the demonstration of phenomena of neurosecretion by morphological techniques does not present much difficulty; the examination of different stages of the evolutionary cycle of these animals and physiological experimentation are yielding information on the functional significance of the phenomena of neurosecretion; but the biochemical approach to this problem is fraught with difficulties. The staining affinities and the histochemical characteristics of the product of neurosecretion do not provide any clue which might guide the biochemist in his attempts at extraction. All the staining affinities of the product of neurosecretion are common to this product and to a great many other products of cellular metabolism. Even the histochemical characteristics of the secretory granules observable in the neurosecretory cells are not of much help; it is now known that the product of hypothalamic neurosecretion is in reality no more than a carrier of the octapeptides which are the so-called posterior pituitary hormones, and the same is probably true of the various products of neurosecretion described in the invertebrates. Besides, the small size of the organs which contain the neurosecretory cells and the necessity for laborious dissection to get them out present appreciable practical complications. It is quite difficult, if not impossible in many instances, to develop a simple biological test applicable to large series of samples and giving a rapid response, so that the biochemist encounters considerable problems in trying to follow the progress of extraction and purification of the hormone under investigation.

These facts explain the scarcity of results available on the biochemistry of the hormones of the invertebrates and insects.

In the literature to which we have had access, there is no information concerning hormonal correlations, an endocrine gland, or phenomena of neurosecretion among the Porifera, the Coelenterata, the Ctenophora, the Nemertinea, the Brachiopoda, or the Bryozoa.

A. Flatworms

The only studies worth mentioning here have been carried out on Turbellaria.

Neurosecretory cells have been demonstrated, by so-called general histological techniques, in the polycladian turbellarians (55); nothing is known about a possible modification of the structure of these cells during the life of the animal.

Ocular regeneration in the planarian *Polycelis nigra* is governed by a humoral mechanism, certain aspects of which have been studied in detail (56, 57). Experiments have shown that the regeneration of the ocellus is induced at a distance by the cerebroid ganglion; the elaboration by this ganglion of chemical substances responsible for this induction ("organisins") must appear probable. If to a medium, in which planarians are raised following excision of the ocella and the brain, are added homogenates of the anterior region of the body containing the cerebroid ganglion, there results a very high percentage of ocular regeneration in the absence of the brain (93% of cases). However, if one adds homogenates of the middle or posterior portion of the body, the percentage of regenerations in the absence of the cerebroid ganglion drops to 10—11%. Organisin, then, appears to be present in the whole body of the animal, but the anterior region, where the cerebroid ganglion is located, seems to contain a very high local concentration.* The presence of this principle appears to be indispensable above all during the first 4 days of regeneration. Some characteristics of this hypothetical substance are known. It is resistant to heating to 60° but is destroyed by heating to 100°; its inductive power is not altered by drying or treatment with alcohol at 70°. Moreover, organisin is devoid of zoological specificity and can diffuse across an agar plate; during centrifugation of homogenates of the anterior region of planarians, the principle goes into the supernatant.

The inductive power of homogenates of the posterior regions of the planarian body, which is normally quite weak, rises when the homogenates are heated to 60° for 2 minutes; this may be due either to the chemical transformation of a compound related to organisin but present normally in the tissues or to the liberation of organisin previously retained in cells whose selective permeability is modified by the rise in heat.

B. Polychete Annelids

Histological data establishing the presence of neurosecretory cells and physiological data proving the existence of humoral correlations among representatives of this class are available, but the nature of presumptive hormonal principles implicated in these phenomena is not known.

Neurosecretory cells have been described in the cerebroid ganglia of numerous species (*58–63*). The application of modern histological techniques has made it possible to distinguish in most of the species several cellular categories, some of which are endowed with a product of secretion which in its staining affinities resembles the product of vertebrate hypothalamic neurosecretion and the product of arthropod protocephalic neurosecretion. An actual secretory cycle of certain cells has been demonstrated (*64–66*). The production of a neurosecretory material by a "cerebrovascular complex" located on the ventral side of the cerebroid ganglion has been described in various Nereidia and in *Nephthys* (*67, 68*).

Furthermore, observation and experimentation have yielded results in support of the mediation of hormonal factors in certain physiological processes:

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* Quite recent experiments [Th. Lender and V. Deutsch, *Compt. rend. acad. sci.* 253, 550–551 (1961)] show, however, that homogenates of chick embryos on the ninth day of incubation contain a principle which can induce the ocular regeneration of *Polycelis nigra* at the same titer as the homogenates of the anterior region of the planarian.

(1) The maturation of male gonocytes is inhibited, in all Nereidia, by a factor produced by the cerebroid ganglion; in certain species, the maturation of female gonocytes undergoes a cerebral inhibition of the same type.

(2) The heteronereidian transformation, in the species which exhibit this phenomenon, is subject to the same inhibition by the cerebroid ganglia. The intervention of these nerve centers has been demonstrated in incontrovertible fashion by experiments involving truncation, ablation of the prostomium, or excision of the cerebroid ganglia, on the one hand, and by experiments on the implantation of cerebroid ganglia into brainless individuals on the other hand (*69–71*). There is no difference in the activity of ganglionic implants according to the sex of the donor, and this action also does not appear to have any sexual specificity. The cerebroid ganglion of epitokous individuals no longer contains this inhibitory factor (*72*). Some experiments suggest the presence in the gonocytes of a factor that antagonizes the inhibitory principle elaborated by the cerebroid ganglia (*73, 74*).

(3) Caudal regeneration is retarded or suppressed by the ablation of the cerebroid ganglia in the Nereidia (*75, 76*), *Nephthys* (*77*), and *Lycastis* (*60*).

(4) The stolonization and sexualization of the Syllidia are inhibited by humoral factors which, according to the results of selective extirpation, are elaborated by the proventricle (*78, 79*).

The existence of endocrine correlations in the polychete annelids, therefore, is well established, but less is known about the chemical nature of the factors that cause them. Even the solubility characteristics of the principles elaborated by the cerebroid ganglia, the gonocytes, and the proventricle of the Syllidia are still unknown, and the histological characteristics of the products of neurosecretion, as already mentioned, do not even have indicative value in this regard. Under these conditions, the endocrine correlations of the polychete annelids can be compared with those of other invertebrates only on the physiological level; suffice it to say here that relations between neurosecretion on the one hand and maturation of the gonocytes on the other occur very frequently.

C. Oligochetes

As in the case of the polychete annelids, the results available on the oligochetes are limited to the description of neurosecretory cells in the cerebroid ganglia and to the demonstration of relations between neurosecretion and growth, reproduction, and regeneration.

Several types of neurosecretory cells have been described in the cerebroid ganglia of the land oligochetes (*80–82*). Some of them undergo very pronounced modifications of structure at the moment of the reproductive period whereas others do not change (*82*).

Ablation of the cerebroid ganglion in animals in full oviposition causes a reduction in the secondary sex characteristics and the arrest of oviposition; the cerebroid ganglion, therefore, appears to elaborate the "cephalic factor" (*83*) that conditions the secondary sex characteristics (*84*).

The participation of the nervous system in regeneration has been shown in a whole series of experiments (*60, 83, 85–88*).

The publications available to us contain no biochemical information on this subject.

D. Hirudinea

The existence of neurosecretory cells in the Hirudinea is well established (*89, 90*) and it is known that the nerve ganglia of these animals contain several types of neurosecretory cells, but no biochemical or physiological data are available which might indicate their functional significance.

E. Sipunculoidea

Neurosecretory cells have been described in three species of Sipunculoidea of the European fauna (*91–93*); there are suggestions of a secretory cycle of these elements during maturation of the gonocytes, but experimental or biochemical studies do not appear to have been devoted to this problem.

Further, extracts of the nephridia of *Physcosoma japonicum* accelerate the rhythm of movements of the isolated nephridium (*94, 95*). The isolation and purification of the compound responsible for this effect does not appear to have been attempted. In another species of *Physcosoma, P. lanzarotae*, an internephridian organ has been described whose extirpation was said to lead to disturbances resembling in certain respects those of adrenalectomy in mammals (*96*).

F. Mollusca

Neurosecretory cells have been described in representatives of all classes of the phylum Mollusca with the exception of the Polyplacophora and the Solenogastra (*97–103*); the functional significance of these elements can be merely the subject of conjecture at this time, except in the Lamellibranchia, where precise experimental results are available. Moreover, an endocrine determinism of the secondary sex characteristics has been proved experimentally in certain pulmonate gastropods, and there is even evidence in favor of a similar mechanism in protobranchiate gastropods. In the cephalopods, the endocrine nature of various organs has been postulated by some authors, but without experimental proof. In all these instances, there is not the least evidence that might give direction to biochemical investigations.

The existence of relations between the neurosecretory cells and the maturation of the gonocytes is rendered probable by the finding that a demonstrable product in the neurosecretory perikaryons accumulates during sexual repose and diminishes during vitellogenesis and at the approach of oviposition (*102, 104*).

Oviposition and ejaculation may be released in the oyster by substances emitted by the gametes of the opposite sex (*105, 106*). The sensitivity of this animal to these principles (gamones of Galstoff, diantline of Nelson and Allison) is increased by the removal of an internal inhibition whose cerebral origin is shown, on the one hand, by the study of the secretory cycle of the neurosecretory cells of the cerebroid ganglion and, on the other hand, by experiments on the selective ablation of this ganglion (*107*).

G. Arthropoda

It is in the phylum *Arthropoda* that the best-known examples of invertebrate endocrine glands are found. The study of hormones in the insects has entered the biochemical stage (cf. Section I), and there is also some information on the biochemistry of the hormones of the crustaceans. Endocrine glands have likewise been described in representatives of other classes of arthropods, but the experimental study of the endocrine correlations is less advanced; we know of no biochemical data on the internal secretions of the Chilopoda, Diplopoda, and Arachnida.

On the basis of anatomical and experimental results, an endocrine function may be assigned to the following structures:

1. A protocephalic neurosecretory pathway, composed of perikaryons whose secretory product has the same staining affinities as the preoptic nucleus of the vertebrate hypothalamus, the axons leading to an organ where this product is accumulated (corpora cardiaca of the insects, sinus gland of the malacostracan crustaceans, cerebral gland of the Chilopoda and Diplopoda, homologous formations of the Arachnida).

2. An endocrine gland with close anatomical connections with this neurosecretory pathway and identified only in insects (corpora allata).

3. An endocrine gland [identified in the pterygote insects (cf. *108* for bibliography), in apterygote insects (*109*), in malacostracan crustaceans (*110*), and in an arachnid (*111*)] of ectoblastic origin and without

direct nervous connection with the other glands of internal secretion; this organ produces the hormonal factor which starts the molting process. This factor has been identified chemically and named ecdysone.

4. An endocrine gland (identified only in the malacostracan crustaceans and called the androgenic gland) which elaborates hormonal factors responsible for the differentiation of the primary and secondary sex characteristics.

5. Neurosecretory cells located in different regions of the nerve centers which produce chromatotropins and, probably, hormones that act on the gonads.

1. *Arachnida*

The protocephalic neurosecretory pathway of the Arachnida has been described in representatives of five orders of this class; in every instance, an organ has been identified to which lead the axons issuing from the neurosecretory perikaryons; on the basis of the histological characteristics of this organ, its endocrine role has been postulated (*112*). There are indications in support of functional relationships between this neurosecretory pathway and sexual activity (*113*). No biochemical studies appear to have been devoted to the hormones that are presumably produced.

A gland which probably is responsible for the secretion of the molting hormone has been identified in the Phalangiidae (*114*).

2. *Diplopoda and Chilopoda*

Only the protocephalic neurosecretory pathway is known in these two classes (*115–117*) we know of no physiological or biochemical data concerning its role or the constitution of the hormonal principles that are elaborated.

3. *Crustacea*

Neurosecretory cells have been demonstrated in some representatives of the Entomostraca, but it is only in the Malacostraca that a real neurosecretory pathway is known; this is connected by perikaryons containing a product of neurosecretion to an organ whose cellular nature has been definitively established by electron microscopy (*118*) and in which the product of neurosecretion accumulates. In the latter, it has been possible, moreover, to demonstrate neurosecretory cells located in various regions of the nerve centers; the axons issuing from some of them establish close relations with the circulatory system (*119, 120*). Electron microscopy shows the similarity between the secretory granules that line the endings of these neurosecretory cells (*118, 121*) and those that are found in the vertebrate neurohypophysis.

With regard to the functional significance of the processes of neurosecretion thus localized, very diverse phenomena have been related to the principles elaborated by the perikaryons filled with products that could be demonstrated by histological techniques, namely metabolic processes, inhibition of molting, development of the gonads, migration of the retinal pigment, and chromatic adaptation.

The biochemical results available at this time are concerned primarily with the chromatotropins (cf. references *122–124* for the bibliography).

Currently, authors accept the classification of the chromatophores of crustaceans on the basis of the color of the pigment which they contain; thus, they distinguish melanophores, xanthophores, erythrophores, and guanophores, the last being white. The chemical data on these pigments have recently been reviewed (*125*).

Two concepts are held at present concerning the chromatotropins. According to the advocates of the unitary theory, the same chromatotropin acts in a different way on the chromatophores of the same species. The supporters of the pluralistic theory hold, on the contrary, that there exist a certain number of specific chromatotropins, each one determining the dispersion or contraction of a given type of chromatophore.

The chemical study of the chromatotropins has encountered a considerable difficulty, namely the small size of the organs which elaborate these products. The studies of Carlson (*126*) showed that the chromatotropins are stable upon desiccation, that they diffuse through cellophane, and that they are not destroyed by boiling for 5 minutes or by *N* hydrochloric acid or sodium hydroxide. According to some authors (*127, 128*), boiling in salt water increases the chromactivity of the extracts, but according to others (*129*) no such effect is seen.

Extraction in an isotonic medium yields preparations with only slight activity on the chromatophores, whereas treatment of the homogenates with distilled water produces very active preparations; this indicates that the hormone-containing granules are surrounded by a semipermeable membrane (*130*).

Some data on the solubility characteristics of the chromatotropins of the crustaceans are shown in Table V, reproduced from Kleinholz (*124*). As Kleinholz pointed out, technical differences make it very difficult to compare the results obtained by various investigators.

Recent investigations (*131*) show that the sinus gland may contain a substance of large particle size endowed with chromactivity; this substance is transformed *in vitro* into molecules of smaller size which

TABLE V

SOLUBILITY CHARACTERISTICS OF THE CHROMATOTROPINS OF THE OCULAR STALK OF DECAPOD CRUSTACEANS[a]

Donor species	Biological test[b]	Solvent	Activity[b]
Palaemonetes sp. (*126, 129*)	*Palaemonetes* E	Ether	0
	Palaemonetes E	100% Ethanol	40%
	Uca M	100% Ethanol	15%
Uca pugilator (*137*)	*Uca* M	100% Ethanol	45%
	Uca M	95% Ethanol	60%
	Uca M	100% Acetone	0
	Uca M	90% Acetone	20%
	Uca M	Ether	0
	Uca M	Petroleum ether	1%
Pandalus borealis (*138*)	*Palaemon* E	Acetone	Used for extraction
	Palaemon E	80% Ethanol	Used for elution from alumina
	Carcinus M	80% Ethanol	0 (?)
Astacus astacus (*139*)	*Astacus* E, X	86% Ethanol	Active on E and X
	Astacus E, X	Ether	Inactive on E and X
	Astacus E, X	Acetone	Active on E
	Astacus E, X	Butanol	Active on E
Crangon crangon	*Astacus* E, X	87% Ethanol	Active on E and X

[a] Reproduced from Kleinholz (*124*).

[b] E = erythrophores; M = melanophores; X = xanthophores.

have different physiological properties and behave differently during electrophoresis. Two compounds may in this way be extracted from freshly dissected sinus glands. One (substance A) causes a strong concentration of the pigment in the erythrophores of *Palaemon serratus* whereas the other (substance alpha) causes only a partial retraction of small erythrophores. Dialysis experiments show that substance A is a larger molecule than substance alpha.

The fact that chymotrypsin (*132*), trypsin (*133*), and papain (*134*) cause inactivation *in vitro* shows that the chromatotropins may be polypeptides.

Attempts at purification of the chromatotropins have yielded a definite enrichment, going from 100 to 200 (*135–137*). More recently, Ostlund and Fänge (*138*) carried out an acetone extraction of the ocular stalks of *Pandalus borealis,* followed by evaporation to a syrupy consistency, washing with ether, solution in acetone, and paper chromatography (ascending method, butanol-HCl). The most active fraction had an R_f of about 0.62, a less active fraction had an R_f of 0.31; spraying with ninhydrin or potassium ferricyanide gave negative results. Chromatography on an alumina column, followed by elution with 85% acetone, evaporation and redissolution in ethanol, second chromatography on an alumina column, elution with 80% ethanol, and concentration under vacuum, yielded very active preparations that had a titer of 100,000 Leander units per milligram (a Leander unit is defined as the smallest quantity of extract which, within 20 minutes, can cause the bleaching of *Leander adspersus* that have been deprived of their ocular stalks. The absorption maximum is located at 2700 Å. A chromatotropin endowed with very similar properties can be obtained, with the same extraction technique, from the anterior third of the larvae of *Tenebrio molitor*. As Ostlund and Fänge pointed out, the absorption maximum in the ultraviolet corresponds to that of numerous aromatic compounds with relatively small molecules.

4. *Insects*

Our knowledge about hormonal regulation in insects is relatively advanced; it is among representatives of this class that the best-known endocrine glands of invertebrates have been identified and the hormonal interactions during postembryonic development have been defined. Therefore, the main outlines of hormonal regulation in insects will be described before the biochemical data concerning the products of neurosecretion are considered. The other insect hormones are considered in Sections V and VI.

a. Hormonal Regulations in Insects: A Survey.

(1) *Hormonal control of molting and metamorphosis.* To our present knowledge, insect hormones are mainly concerned with developmental physiology, i.e., with molting and metamorphosis (for reviews cf. *108, 140–143*). The biochemistry of insect metamorphosis is dealt with extensively in Chapter 4 of this volume; thus there will be some overlapping. In this chapter, insect hormones and their main physiological and biochemical effects will be covered; in Chapter 4 of this volume a detailed picture of the biochemical events during insect metamorphosis are given, irrespective of whether they are direct or indirect effects of the hormones.

A short account of hormonal actions in molting and metamorphosis follows: Three hormones are involved—the brain hormone, produced in the neurosecretory cells (see below); the prothoracic gland hormone, and the juvenile hormone, produced in the corpora allata. The brain hormone stimulates the prothoracic glands, which in turn produce and secrete their hormone, ecdysone. It is the general "molting hormone" and induces all processes that are preparatory to a molt and finally the molt proper. If the juvenile hormone is secreted simultaneously, this will be a larval molt; if the juvenile hormone is lacking, the result is a pupal or imaginal molt (see also Fig. 1 in Chapter 4 of this volume).

Of these three hormones, only the brain hormone is classified among the neurosecretory hormones. Ecdysone and juvenile hormone belong to the third group and are covered in Section V.

(2) *Other processes controlled by neurosecretory hormones.* Diapause (*144*), i.e., the arrest of development on a certain stage for a certain time, may also be controlled by a hormone arising in the subesophageal ganglion. Some authors have demonstrated myotropic activity of extracts from various organs, mainly corpora allata and corpora cardiaca. Color change is also controlled by hormones, at least in some species, though the action is not as distinct as in crustaceans. It may be mentioned that extracts from insect heads or isolated corpora allata are highly active on the chromatophores of crustaceans (contracting the red chromatophores). Color change and myotropic activity are due, according to Gersch, to neurohormones (see below).

b. The Prothoracotropic Hormone of the Brain.

(1) *Historical.* Hormonal activity of the central nervous system was discovered by Kopeč in 1922 (*145*). The problem was reinvestigated by Wigglesworth (*149, 150*) in the Hemiptera and by Kühn and Piepho (*146–148*) in the Lepidoptera; it was established that removal of the brain inhibits molting, and reimplantation caused the operated animals, the "permanent larvae," to pupate. Later it was shown that the brain hormone is produced in the neurosecretory cells and that not only pupal molt, but also imaginal development, is initiated by the brain. This was most easily studied in the large American silkworm *Hyalophora cecropia* (formerly *Platysamia cecropia*) which has a pupal diapause; termination of diapause, i.e., imaginal development, is initiated by hormonal secretion of the neurosecretory cells (*151, 152*).

The prothoracotropic hormone is often referred to as "the brain hormone" in spite of the fact that there are presumably several neurosecretory hormones produced by the neurosecretory cells (see below). The target organ of the brain hormone is the prothoracic gland (*153*) which is stimulated to produce ecdysone; recently, a direct synergistic effect on the tissues has been claimed (*154*).

The same interrelationship between brain, prothoracic gland, and molting holds for all insect orders so far investigated (for reviews see *140–143*).

(2) *Neurosecretory cells of the brain.* Neurosecretory cells have been discovered in the cerebroid ganglia of all the insects studied in this regard (for reviews cf. *102, 108, 140–143*). A particularly important

group is located in the pars intercerebralis of the protocerebron of the pterygote insects. The number of neurosecretory cells of the pars intercerebralis is variable: in general, the hemimetabolic insects have very small but numerous cells, the holometabolic insects have few but very voluminous cells. The product of secretion of these cells stains intensely with chromic hematoxylin by the method of Gomori and with paraldehyde fuchsin. Therefore, it has staining affinities comparable to those of the product of secretion of the hypothalamic-neurohypophyseal pathway of the vertebrates, but this obviously does not imply the least similarity of the hormonal principles elaborated in the two instances. The axons that issue from the perikaryons of the pars intercerebralis lead to the corpora cardiaca, forming the nervi corporis cardiaci 1. They innervate the corpora allata in all the pterygote insects with the exception of the Ephemeroptera, where this innervation is provided by the subesophageal ganglion. The product of secretion of the cells of the pars intercerebralis is detectable along the nervi corporis cardiaci 1, in the corpora cardiaca, and, in some insects, in the corpora allata. A secretory cycle of the cells of the pars intercerebralis during postembryonic development has been described in insects belonging to various orders.

Neurosecretory cells have been demonstrated, in some insects, in regions of the cerebroid ganglia other than the pars intercerebralis; the morphological and physiological data concerning these elements are still fragmentary.

(3) *Chemistry of the brain hormone.* Though the biological activity of the brain could be demonstrated by transplantation experiments, efforts to extract and isolate "the" brain hormone were long unsuccessful. The first active extracts were obtained by Kobayashi and Kirimura (*155*). Starting from 8300 isolated brains, these authors obtained an extract which was purified through solvent extractions.

The purified extract was assayed in *Bombyx* pupae from which the brain had been removed (permanent pupae) and proved to be active, i.e., it initiated development. This was taken as evidence that the prothoracic glands had been stimulated to produce the molting hormone, ecdysone. The purified extract has not been tested for ecdysone which would produce the same biological effect; but the method of extraction virtually rules out the possibility of contamination with ecdysone.

Recently, the isolation of the "brain hormone" in crystalline form has been reported (*155a*). The substance has been identified as cholesterol (*155b*). It is difficult to imagine that cholesterol, being present in many insect tissues in fairly large quantities, can serve as a hormone. An independent reinvestigation of this problem seems advisable.

Gilbert and Schneiderman (*156*) have obtained evidence that ether extracts from male *H. cecropia* adults contain some brain hormone together with the juvenile hormone. Diapausing animals receiving these preparations developed into second pupae. That they gave rise to second pupae instead of to adults is due to the juvenile hormone; but that they developed at all is the effect of the brain hormone. Brainless pupae responded in the same way, whereas isolated abdomens (lacking the prothoracic gland) showed no development. It is impossible at the moment to identify both preparations, though the solubility characteristics published by Kobayashi and by Schneiderman are essentially the same.

Recently, two other preparations with brain hormone activity have been described. Ichikawa has obtained preparations that are water soluble (*157*) and are active in brainless pupae. Furthermore, Gersch (*158*) has claimed that the "neurohormone D" has brain hormone activity. He used a different assay method: into ligated *Calliphora* larvae abdomens, an immature ring gland was implanted and simultaneously the active material was injected. The observed effect is the puparium formation of the larval abdomen. In Gersch's experiments, a nervous connection between brain and ring gland seemed not to be necessary; this is at variance with the observation of Possompés (*159*). The assay method of Gersch seems rather complicated since he had to use transplanted ring glands; some side effects of either the transplant or the injected extract (which may contain minute amounts of ecdysone) cannot be excluded. It is impossible to say at the moment how Gersch's preparations compare with those of Ichikawa.

Even more confusion about the chemical nature of the brain hormone has been brought about by the reports of L'Hélias (*160, 161*) on the occurrence of pteridine derivatives in insect brains; she claimed that they are related to the brain hormone. There is little doubt that pterines are present; they can be found in nearly any insect tissue (*162, 163*). Experimental evidence for their role in the control of metamorphosis is lacking; on the contrary, direct tests in brainless diapause pupae of *Hyalophora cecropia* showed no effect [Williams, private communication, cited from (*141*); see also Dupont-Raabe (*164*) and de Lerma *et al.* (*165*)]. In a reinvestigation, Fischer *et al.* (*165a*) have shown that some pterines have the same R_f values, in paper chromatography, as the neurohormones C and D (see below). By more elaborate techniques, the active substances could be separated from the pterines.

(4) *Control of the activity of neurosecretory cells.* There seems to be a correlation between nervous activity, as demonstrated with electrophysiological methods, and neurosecretory hormone production in the pars intercerebralis (*166*). During pupal diapause, the brain shows no spontaneous electric activity. Only after termination of diapause by

proper temperature stimuli does the brain resume electrical activity and hormone production.

c. Other Hormones Produced by Neurosecretory Cells. It has already been pointed out that neurosecretion is a prominent feature in lower animals, and it is often difficult to correlate the histological picture of neurosecretion and the physiological action of the "neurohormones." In insects, there are four prominent effects of neurosecretory hormones: prothoracotropic activity (see above); induction of egg diapause in *Bombyx;* myotropic activity which influences heart, gut, and Malpighian tubules; and melanotropic effects.

(1) *The diapause hormone of Bombyx mori.* In the commercial silkworm, *Bombyx mori,* monovoltine, bivoltine, and polyvoltine races are known. In the bivoltine race, every second generation enters egg diapause. The stimuli are photoperiod and temperature during larval life of the females (*167*). If the subesophageal ganglion of a female conditioned to lay hibernating eggs is transplanted to another female conditioned to produce nondiapause eggs, the operated female will produce diapausing eggs (*168–171*). The active principle has been extracted from subesophageal ganglia (*172*) or whole heads and assayed in multivoltine *Bombyx* females. The purification of this factor is under way (*173*).

The physiology of egg diapause in *Bombyx* is presumably a very special case; it has a parallel perhaps in the recently discovered hormone-like factors that control behavior (general activity, diurnal rhythm) in the cockroach and which are also secreted by the subesophageal ganglion (for review, see *174*). In general, diapause seems to be a phenomenon that can be controlled by various factors, including the brain hormone (e.g., *H. cecropia,* see above), the juvenile hormone [e.g., in the Colorado beetle, *Leptinotarsa decemlineata*) (*175*), in the rice stem borer (*176, 177*), and perhaps also in the sawfly *Cimbex americana*], and other factors (see Lees, *144*, for review).

Myotropic activity by extracts from various organs has been described very often (see Koller, *178, 179,* for review), but only in a few cases has the active principle been investigated and distinguished from widespread factors like acetylcholine, adrenaline, etc. Cameron (*180*) detected in the cockroach nerve a dihydroxyphenol that is believed to be different from substances described so far. The biological system is, however, very complicated, the pericardial cells being involved (*181*). The hypothesis has been put forward that the corpus cardiacum substance acts on the pericardial cells, which in turn release some pharmacologically active, heart-accelerating factor.

The neurohormones investigated by Gersch (*182–187*) act on the color change as well as on the heart beat. Gersch *et al.* distinguished several factors. One was identified as acetylcholine; others, i.e., the so-called neurohormones C and D, are different from all known neurohumors. They can be assayed (*a*) on the melanophores of *Corethra* larvae, (*b*) on the heart beat of the same animal, (*c*) on the color change of the stick insect, *Carausius morosus,* (*d*) on the frog heart preparation of Straub. Attempts at isolation yielded purified preparations; the authors finally described a crystalline "neuro-hormone D_1" which did not melt below 350°. The first crystalline preparation (*185*) was obtained in a yield of 2 mg. from 320 cockroach nerve cords; later the authors described the isolation of the same substance in a yield of about 50 μg. per 3200 nerve cords (*187*).

This discrepancy has not been explained, and criteria for purity are not given. The substance is believed to be a peptide. A proper evaluation of these reports will be possible only when more details on the chemistry have been published.

Melanotropic activity has been attributed to some of the preparations already discussed (*161, 164, 182*). The color change in *Dixippus* and in *Corethra* larvae seems to be under control of neurohormones. Extracts from insect heads, mainly from corpora allata and corpora cardiaca, are active also in the crustaceans (*164, 188, 189, 190*).

Crustacean and insect color-change hormones may be identical. According to Gersch (*190a*), the four active substances detected in crustaceans and separated by paper electrophoresis by Carlisle and co-workers (*190b*) have been identified as *N*-acetylcholine, serotonin, and the neurohormones C and D of insects.

V. Hormones Elaborated by Tissues Other Than the Nervous System

Occasional observations, some of which have been mentioned briefly in Section IV, suggest that hormonal actions may originate in organs other than the nervous system in certain representatives of the heterogeneous grouping of the worms. More precise data are available on the mollusks, the arthropods, and the protochordates.

A. Mollusks

In the gastropods, the existence of sex hormones which condition the state of the secondary sex characteristics is suggested by classic observations which demonstrated a chronological concurrence of the development of the gonads on the one hand and that of the secondary sex characteristics on the other. The observations on parasitic castration point in the same direction, and the same is true of the rare observations of hermaphrodism in normally gonochoric species (*191*). In the Limacidae and the Arionidae, ablation of the gonad in sexually mature individuals is followed by a very marked regression of the albumin gland and of the glands attached to the spermoviduct. Homo- or heteroplastic grafts of the mature gonad into the immature animal cause pronounced hypertrophy of the glands attached to the genital tracts. The same hypertrophy occurs if these glands, removed from immature animals, are implanted into adult animals. These findings demonstrate the reality of a humoral mechanism of conditioning of the genital tracts, the active compound being produced by the gonad (*191*).

Mention may be made of the paucity of experimental data concerning the organs for which an endocrine role has been postulated in the cephalopods. The surgical extirpation of the epistellary body of octopods (*Octopus, Loligo*) causes a considerable degree of hypotonicity in the animal (*192*); the ablation of the branchial bodies had as a consequence an arrest of growth (*193*); the functional significance of the stalk glands is still unknown (*194, 195*).

B. ARTHROPODS

An endocrine gland which is probably involved in the conditioning of molting has been demonstrated in the Phalangiidae (*114*); this organ has not yet been subjected to experimental study. In other Arachnida, in Diplopoda, and in Chilopoda, endocrine glands distinct from the nervous system have not yet been identified with certainty. However, positive results are available concerning the endocrine glands of the crustaceans and insects.

1. *Crustaceans*

A molting gland has been described in all the malacostracan crustaceans, with the exception of the Syncarida (*196, 197*). The involvement of this organ in the causation of molting, asserted on the basis of cytological data (*196*), has been demonstrated by experiments with selective ablation and implantation (*198, 199*). Extraction carried out according to the techniques which have led to the isolation of ecdysone (cf. below) yielded a compound which has the same biological properties as ecdysone and is closely related, if not identical with it, chemically (*200, 201*).

An androgenic gland that produces hormonal principles that condition at the same time the state of the gonad and of the secondary sex characteristics has been described in the amphipod *Orchestia gamarella* (*202*); later, it was identified also in the Leptostraca (*203*), the Isopoda (*204*), and the Decapoda (*205*). The role of this organ in differentiation and in the maintenance of the secondary sex characteristics has been demonstrated experimentally, but no chemical information is available on the hormone that is produced.

2. *Insects*

The review of the hormonal correlations in insects, presented above, indicates that three hormonal principles enter into consideration here, namely ecdysone, elaborated by the prothoracic gland (ventral gland of hemimetabolic insects), the juvenile hormone, produced by the corpora allata, and a gonadotropic factor, also produced by the corpora allata.

a. Ecdysone, the Hormone of Prothoracic Glands. The brain hormone stimulates the prothoracic glands to produce a second hormone. This has been named ecdysone (derived from ecdysis = molt) because it is responsible for the initiation of molting, especially initiating the epidermal changes. Ecdysone was isolated in pure, crystalline form by Butenandt and Karlson (*206*) in 1954; it was the first insect hormone obtained in crystalline form.

(1) *History.* The physiological action of the prothoracic gland hormone in *Calliphora* was demonstrated in 1935 by Fraenkel (*207*), long before the prothoracic glands had been recognized as the center of production. Following the work of Fraenkel, Becker and Plagge (*208*) obtained the first extracts active in the *Calliphora* assay. The prothoracic glands were then rediscovered by Fukuda (*153, 209*), who demonstrated their role in the determination of the pupal molt; their importance for imaginal development was established by Williams (*152*). These authors did not succeed, however, in getting active extracts. The chemical work of Becker (*210*) was continued quite independently by Butenandt and Karlson (*206*), who obtained crystalline ecdysone in 1954.

(2) *Bioassay.* There are a number of bioassays for ecdysone. The easiest and most sensitive is the *Calliphora* assay to be described below. Wigglesworth (*211*) used ligated *Rhodnius*, which molts after the injection of ecdysone. The dose needed is about 100 times larger than in *Calliphora*. Also isolated abdomens of diapausing *H. cecropia* pupae had been used (*212*); they do not contain any known endocrine gland and are therefore in some respects preferable to other systems. On the other hand, they are not very sensitive; initiation of development can be achieved only with 1000 *Calliphora* units (C.U.). Another method made use of spermatocytes cultured *in vitro* (*209*). In this assay, however, some protein component was active, but crystalline ecdysone failed to give positive results (Williams, personal communication cited from reference *141*). A possible interpretation is that the ecdysone may be bound to some protein in the hemolymph. Our experiments showed, however, that the ecdysone activity of hemolymph is freely dializable. It seems

more likely, therefore, that the spermatocyte assay measures some active principle other than ecdysone.

Numerous other effects of ecdysone have been observed (see Table VI) that may be used as a basis for bioassay, but none has been worked out in detail.

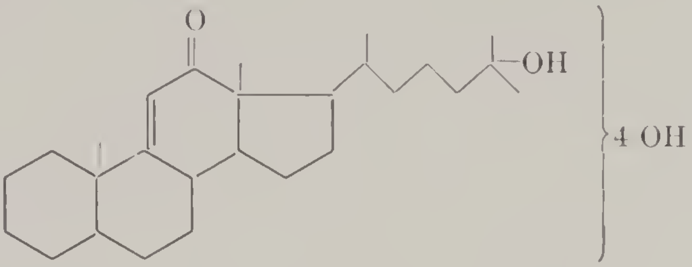
In our experience, the best bioassay is the *Calliphora* assay introduced by Fraenkel and by Becker and Plagge and modified by Karlson. Fully grown larvae at about 10–15 hours before spontaneous pupation are ligated; those animals pupated only in the head part are selected and their surviving abdomens are used as the test preparation. The selection guarantees a good sensitivity and a uniform response, in this respect, the *Calliphora* assay is superior to all other biological methods in the field. Some data have been published (*141*).

Extraction and purification. Extracts containing the prothoracic gland hormone can be obtained from many sources. Systematic studies have not been made, but preliminary experiments have demonstrated that pupae of Lepidoptera (*Bombyx, Hyalophora cecropia, Cerura vinula*) and of Diptera (*Calliphora*) yield active extracts. In the latter case α-ecdysone has been identified as the active principle. Also, imagoes of the desert locust can be used as a source. Our observations indicate that the age of the pupae has much influence on the activity of the crude extract.

The method of extraction has been worked out in detail with *Bombyx*. Pupae are extracted with methanol; the solution is concentrated to small volume and extracted with butanol. The butanol layer is washed with acid and alkali and evaporated to dryness. Alternatively, the concentrate of the original methanolic solution can be extracted with petroleum ether in order to remove the greater part of the lipids before the butanol extraction is made. The next purification steps, which involve chromatography and countercurrent distribution, should be controlled by bioassays.

Ecdysone has been obtained in a yield of about 50 mg. per ton of starting material. The proportion of the biological activity which can be obtained as crystalline α-ecdysone depends on the quality of the pupae (age ?). From the side fractions, another substance with hormonal activity has been obtained in crystalline form; there are some indications that even more related substances are present. This remains to be investigated.

Chemical properties of prothoracic gland hormone. The crystalline material isolated from *Bombyx* pupae and having the activity of prothoracic gland hormone has been named α-ecdysone. It has a melting point of 241–244°, a characteristic ultraviolet (UV) absorption maximum at 244 mμ, and an infrared absorption band at 6.02 μ. The preliminary empirical formula is $C_{27}H_{44}O_6$; one oxygen is present in a keto group in conjugation to a double bond, and the five others are in hydroxyl groups. Dehydrogenation to phenanthrene derivatives showed that ecdysone belongs to the steroids. The accompanying partial formula (I) has been given (*212a,b*).



(I)

By countercurrent distribution, a second active substance, β-ecdysone, has been separated (m.p. 177–178°). Its spectral characteristics are very similar to those of α-ecdysone. β-Ecdysone is only half as active as α-ecdysone.

On paper chromatograms, ecdysone can be detected either by the photoprint method or by spraying with strong acids or with strong alcoholic alkali, followed by inspection in UV light for fluorescence.

Metabolism of ecdysone. There is little doubt that ecdysone is inactivated within the animal. In many biological experiments, the effect of a single injection lasts only for several hours. (Multiple injections have been tried in some cases, but often result in a high mortality.) Furthermore, *Bombyx* imagines contain only about one-tenth the amount of ecdysone activity of fresh pupae (*213*). For a more detailed study of variations in ecdysone content, see (*213a*).

Details of the metabolism are completely unknown. Also, the inactivation products have not been identified. Tracer methods could clarify this point, but labeled ecdysone has become available only recently (*213b*).

The biogenesis of ecdysone has been partly elucidated. It is formed from cholesterol, as shown by conversion of H^3-cholesterol into radioactive ecdysone in *Calliphora* (*213c*).

Physiological action of ecdysone. Since the physiology of ecdysone is mainly the physiology of insect metamorphosis, it will be dealt with only briefly. For details, the reader is referred to Chapter 4 of this volume. Table VI lists the effects observed after injection of ecdysone. Various other effects supposed to be due to ecdysone, as revealed by biological experiments (ligation, parabiosis, implantation, etc.), are not incorporated. The table is organized according to zoological systematics; our discussion will follow the developmental line.

TABLE VI
The Action of Ecdysone in Various Insects

Order	Species	Preparation	Type of action	Dose necessary (in Calliphora units)	References
Diptera	*Calliphora erythrocephala*	Ligated larval abdomen	Puparium formation	1	(*141*)
	Drosophila melanogaster	*lgl* larvae (defective ring gland)	Puparium formation	3	(*214*)
	Chironomus tentans	Late larvae	Activation of genes	0,001	(*215*)
Lepidoptera	*Ephestia kühniella*	Ligated larval abdomen	Pupation	20–50	(*216*)
	Galleria mellonella	Abdomen	Pupation	20–50	(*216*)
	Cerura vinula	Ligated larval abdomen	Color change	13–66	(*217*, *218*)
	Hyalophora cecropia	Brainless, diapause pupae	Initiation of development	825–1330	(*212*)
	Samia walkeri	Isolated abdomen of diapause pupae	Initiation of development	1330	(*212*)
Hymenoptera	*Cimbex americana*	Diapause larvae	Initiation of development	660	(*219*)
Hemiptera	*Rhodnius prolixus*	Decapitated larvae	Molting	66–100	(*211*)
Isoptera	*Kalotermes flavicollis*	Normal worker larvae	Molting	3	(*220*)
Neuroptera	*Sialis lutaria*	Hibernating larvae	Pupation	50	(*221*)
Orthoptera	*Cryptocercus*	Normal larvae	Induction of sexual cycles in symbionts	100–1000	(*222*)

Larval molting is generally believed to be due to the simultaneous action of ecdysone and juvenile hormone (see below). There are, however, only very few direct experiments on larval molting. In most cases it is necessary to eliminate the animal's own hormone-producing system (e.g., by ligation) in order to obtain clear-cut results, and only few organisms are suited to experiments on the initiation of larval molts. Many species need a certain feeding period ("indispensable feeding period") before they are ready to molt, and after ligation the gut contents often gives rise to high mortality.

Rhodnius, a tropical bug, needs only one full blood meal in every instar, and Wigglesworth has done numerous experiments on the determination of molting and metamorphosis. In this case, it has been experimentally proved that ecdysone can initiate molting in ligated larvae (*211*). These animals, however, being deprived of their corpora allata, need a source of juvenile hormone in order to perform a true larval molt.

Another example is the induced nymphal (larval) molt in termites (*220*). Termite workers (pseudergates) can be made to molt within 10 days after injection of ecdysone; in this case, the normal animals do not molt in the experimental period, so that elimination of the glands is not necessary. The dose needed is rather low (about 3–5 C.U.). Higher doses are not toxic and do not influence the character of the molt, i.e., they do not produce imaginal differentiation. It is remarkable that in these experiments ecdysone has proved to be active also when administered by feeding.

Strictly speaking, the transformation of *Calliphora* larvae into a puparium is also a specialized larval molt rather than a pupal molt; this process will be dealt with in detail in Chapter 4 of this volume.

True pupation of caterpillars has been achieved by injection of ecdysone in *Ephestia* (*216*) and in *Cerura vinula* (*218*). In *Ephestia*, good results were obtained only with animals ligated shortly before spontaneous pupation. It is presumed that the hormone is inactivated in the insect and can exert its action only for a short time, under some conditions not long enough to initiate molting (see also page 274). Other species have not been investigated in detail.

Metamorphosis, i.e., induction of an imaginal molt, is the basis of assay methods in *Rhodnius* and in *H. cecropia;* in the latter case, diapausing brainless pupae or isolated abdomens are used. A single injection of a rather high dose of ecdysone suffices to bring the development to completion. For details, see Chapter 4 in this volume. Other work is listed in Table VI. Of special interest in respect to the mode of action of hormones is the induction of puffs in salivary gland chromosomes (Clever and Karlson, *215*). The induced puff is on the first chromosome (locus I.R. 18) and is characteristic for early prepupae. The hypothesis has been advanced that hormonal induction of development is, in fact, the activation of certain genes and the "release of information" therefrom.

Another peculiar effect has been observed: the induction of sexual cycles in symbionts (*222, 223*). The unicellular symbionts of the wood-feeding roach *Cryptocercus* propagate asexually; only during the molting cycle of the insect, do they undergo gametogenesis, autogamy, and self-fertilization. This could be induced by injection of ecdysone. It is remarkable that the symbionts are even more sensitive to ecdysone than the host: they respond to a dose of 100–250 Calliphora units whereas the roaches show no signs of molting with such a low dose.

b. The Juvenile Hormone. The juvenile hormone is produced by the corpora allata. It determines the larval character of a molt: only in the presence of (active) corpora allata, will a larva molt to become another larva; otherwise it will become a pupa or adult. The biological role of the corpora allata has been established through the work of Wigglesworth (*150*), Bounhiol (*224*), Piepho (*225*), and others. Attempts to extract the active principle were long without success until C. M. Williams detected a very rich source in the adult male moth of *Hyalophora cecropia* (*226*).

(1) *Bioassay.* Several biological effects have been proposed as method of bioassay. The production of supernumerary larval instars or of intermediates between larvae and pupae in moth (e.g., the wax moth *Galleria*) is too insensitive. Pupae can be forced to produce a second pupa or to give intermediate forms; by this method, Williams detected the activity of extracts. Even more sensitive is the wax test developed by Gilbert and Schneiderman (*227*); in this test, the material is applied locally and its local action is observed.

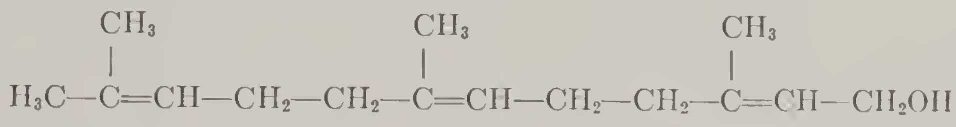
Wigglesworth (*228*) investigated several species of different insect orders in respect to a possible bioassay. *Rhodnius* gave good results with a local test on the cuticle; the treated segments showed larval instead of imaginal characters. Also, the genitalia remained larval. The same method was successful in *Tenebrio* pupae; in this insect, the pupal cuticle is pale yellow, the imaginal is dark brown, and the patches of pupal cuticle in the imago are easily detected on the imaginal abdomen. A modification of this assay is used in our laboratory; we inject under the cuticle a small known amount of the material to be assayed and get a response which can be quantitated (as percentage of positive animals) (*229*).

(2) *Extraction, purification, and chemistry of the juvenile hormone.* The extraction method of Williams (extraction with ether or with alcohol plus ether) has become a standard procedure in work with juvenile hormone. Purification procedures have not been reported in detail;

countercurrent distribution and chromatography on alumina has apparently yielded a "highly purified material" (*230*).

A point of considerable comparative interest is the fact that a large variety of biological materials yielded active extracts. Not only arthropods—insects and crustaceans—but also vertebrate tissues as well as certain bacteria contain some activity as revealed by the bioassay. Table VII, taken from Schneiderman (*231*; cf. *231a–c*), gives a survey of several phyla.

Also, quite different tissues yielded active extracts. We have found that the feces of the yellow mealworm, *Tenebrio molitor,* contain some juvenile hormone activity (Karlson and Schmialek, *232*). The substance responsible has then been identified as farnesol (*233*):



$$H_3C-\overset{\overset{\large CH_3}{|}}{C}=CH-CH_2-CH_2-\overset{\overset{\large CH_3}{|}}{C}=CH-CH_2-CH_2-\overset{\overset{\large CH_3}{|}}{C}=CH-CH_2OH$$

It is unlikely, however, that farnesol is identical with insect juvenile hormone: the activity is not as high as would be expected (*229*). On the other hand, it may well be that farnesol or related compounds which are common to virtually all organisms are responsible for the juvenile effects of thymus or adrenal extracts; farnesol pyrophosphate is a precursor of the sterols and may be present in many tissues.

Williams (*230*) describes some preliminary findings of methylene lactone groups in his purified material. It is difficult to evaluate these reports since no criteria of purity are given for the "purified material." On the other hand, the activity of farnesol in juvenile hormone assays may point to a chemical relationship with the isoprenoid compounds, a relationship which is not excluded by the known chemical properties of farnesol.

(3) *Physiological effects of the juvenile hormone.* The action of juvenile hormone has mainly been studied with the classic methods of endocrinology, i.e., extirpation and transplantation of the corpora allata. It would be of interest to know more about the activity of juvenile hormone preparations in insects, but only few data are available.

Extirpation of the gland results in precocious pupal and imaginal molts. Implantation gives rise to supernumerary larval instars in a variety of species and orders [*Lepidoptera* (*224, 225*), *Coleoptera* (*234*), *Hemiptera* (*150, 235, 236*)]. The effect is quite variable, and often intermediate forms between larva and pupa or pupa and adult have been obtained; the latter is also true for work with purified juvenile hormone extracts.

Some other effects due to juvenile hormone are the color change in

TABLE VII
JUVENILE HORMONE ACTIVITY OF ETHER EXTRACTS OF BIOLOGICAL MATERIAL
(*a*) FROM ANIMAL SOURCES (*231, 231a*)

Phylum	Class	Species	Activity
Porifera	Desmospongiae	*Microciona prolifera*	0
		Cliona celata	0
Cnidaria	Hydrozoa	*Pennaria tiarella*	+
		Tubularia crocea	+
		Pelmatohydra oligactis	+
		Gonionemus murbachii	+
		Physalia physalis	+
	Anthozoa	*Alcyonium carneum*	+
		Metridium senile	0
Platyhelminthes	Turbellaria	*Cura foremani*	0
Rhynchocoela	Anopla	*Cerebratulus lacteus*	0
		Micrura caeca	+
Ectoprocta	Ctenostomata	*Alcyonidium verrilli*	+
	Cheilostomata	*Bugula flabellata*	+
Annelida	Polychaeta	*Chaetopterus variopedatus*	+
		Glycera dibranchiata	+
		Nereis virens	+
	Oligochaeta	*Lumbricus terrestris*	+
Arthropoda	Insecta	Numerous Lepidoptera	+
		Tenebrio molitor (Coleoptera)	
		(larvae)	+
		(adults)	0
		Sarcophaga bullata (Diptera)	
		(larvae)	0
		Neodiprion lecontei (Hymenoptera)	
		(diapausing prepupae)	0
		Apis mellifera (Hymenoptera)	
		(winter workers)	0
	Malacostraca	*Uca pugilator*	+
		Orconectes immunis	0
		Homarus americanus	
		(eyestalks)	++
		Carcinides maenas	
		(eyestalks)	++
		Crago septemspinosus	+
		Palaemonetes vulgaris	0
		Upogebia affinis	0
	Merostomata	*Limulus polyphemus*	0
	Arachnida	*Pholcus* sp.	+
Mollusca	Pelecypoda	*Mya arenaria*	0
		Venus mercenaria	+
	Gastropoda	*Deroceras agreste*	+

TABLE VII (*Continued*)

Phylum	Class	Species	Activity
Echinodermata	Holothuroidea	*Thyone briareus*	0
		Leptosynapta inhaerens	+
	Echinoidea	*Arbacia punctulata*	0
Enteropneusta	Balanoglossida	*Saccoglossus kowalevskii*	+
Vertebrata	Mammalia	Rat (new-born)	+
		Cow	
		(various organs)	+
		(thymus)	++

(*b*) FROM MICROORGANISMS[a]

Species	Type of extracts	Per cent positive tests	Activity
Ochromonas malhamensis	ether	45	++
Tetrahymena pyriformis	ether	18	+
Bakers' yeast (fresh)	ether	25	+
Brewers' yeast (dried)	ether	100	++
Micromonospora sp. (mycelium)	chloroform	65	+
Escherichia coli (cell-free broth)	ether	75	+++
Proteus spp. (whole broth)	ether	75	+++

(*c*) FROM PLANT SOURCES

Species	Activity
Wheat	
(bran, ether extract)	+
(germ oil)	0
Soybean (oil)	0

(*d*) CHEMICAL COMPOUNDS OF NATURAL ORIGIN (*231c*)

Compound	*Tenebrio* units/mg.
Farnesol	30
Farnesyl phosphate (oily suspension)	(40)
Farnesyl pyrophosphate	
(oily suspension)	0
(aqueous suspension)	0
Farnesyl methyl ether	38,000
Farnesyl diethylamine	35,000

[a] A large number of other microorganisms were tested and found to be inactive (*231b*).

Locusta (*237*), especially in the transition from solitary to gregarious forms, and the type of cocoon spun by *Galleria* larvae (*238*). Caste determination in termites is also controlled by the corpora allata (*239*); for gonadotropic activity, see below.

c. The Gonadotropic Hormone (Yolk Formation Hormone, Metabolic Hormone) of the Corpora Allata. Soon after the first demonstration of the endocrine function of the corpora allata, Wigglesworth discovered their role in reproduction (*150*). Extirpation of the corpora allata inhibits egg ripening in the ovaries; after reimplantation, the eggs begin to develop. The same has been found in most insect species with the exception of the order Lepidoptera, where egg ripening and yolk formation is entirely independent of the corpora allata.

The function of the corpora allata in respect to ovary stimulation seems to be controlled by some neurosecretory hormones from the brain. Thomsen (*240*) has found that some nerves coming through the corpora cardiaca are apparently involved in this control, at least in *Calliphora*.

It has been claimed by various authors (see *142, 143*) that the "yolk formation hormone" of the corpora allata is in fact the juvenile hormone. Their evidence is rather indirect; it stems from transplantation experiments of "juvenile," "active," and "inactive" glands. It must be kept in mind that the activity of a gland may be controlled by nervous or humoral factors; therefore the status of activity of a transplanted gland may be different in the host and in the donor.

The opposite view is taken by Lüscher *et al.* (*239, 241*). Their theory of caste determination in termites operates with a juvenile hormone and a gonadotropic hormone, which are secreted independently. Determination into one or the other direction depends on the time and amount of the different secretions.

Also, the metabolic rate is independent from the presumed juvenile hormone concentration (*242*). This evidence is, however, also indirect; it assumes that the yolk-formation hormone stimulates metabolism.

(1) *Bioassay.* No reasonable bioassay for the yolk-formation hormone activity has been worked out. A number of effects have been observed: development of ovaries in the female of *Calliphora* (*243*) and *Rhodnius* (*150*) and in pregnant *Leucophaea* (*244*), and changes in the collateral glands of the cockroach (*245*). Larsen and Bodenstein (*246*) have published some observations on egg development in mosquitoes, which seemed to be the basis of a bioassay. We have tried to reproduce their experiments, but we have not observed egg development after injection of various extracts (*247*).

Since purified samples of juvenile hormone are available, it would be obvious to try them in an assay system for gonadotropic activity. Most experiments of this kind have been negative; only recently, Wigglesworth (*248*) has obtained positive results with farnesol, the active principle of *Tenebrio* excreta (*232, 233*), and with some derivatives of farnesol (*248a*). If this finding could be extended to other species, the identity of yolk-formation hormone and juvenile hormone would be established. However, there is only little stimulation of egg ripening in *Calliphora* after injection of farnesol (*247*).

(2) *Physiological effects of the yolk-formation hormone.* The main effect on yolk formation has already been discussed. It may be mentioned that in roaches the accessory glands which produce the material of the oothecae are also under the control of this hormone (*245, 249*).

The effect on yolk formation may be indirect, arising from a general stimulation of metabolism. Several authors found a decrease of basal metabolism (O_2 consumption) after allatectomy (*250–252*). There is an influence on fat metabolism (*253*), on the proteases (*254*), on water balance (*255*), and perhaps on some other metabolic processes (nitrogen metabolism and phosphorus metabolism) (*256*). The metabolic rate has been taken as indicator of metabolic hormone production; it does not coincide with the presumed juvenile hormone formation (*242*).

(3) *Control of hormone production.* The function of the corpora allata in respect to ovary stimulation seems to be controlled by some neurosecretory substances from the brain. Thomsen has found that some nerves coming through the corpora cardiaca and carrying neurosecretory material are apparently involved.

C. PROTOCHORDATA

This name covers two subphyla of the Chordata, the Cephalochordata, exemplified by *Amphioxus*, and the Urochordata, of which the tunicates are the most typical representatives. To these are sometimes added the Hemichordata or Enteropneustes, which, however, are more appropriately considered to be another phylum since only the first two groups contain an outline of a dorsal chord—in the adult state of the Cephalochordata and in certain larval stages in the Urochordata. Certain morphological features of the Protochordata relate them to the vertebrates, others to the echinoderms, the group of invertebrates nearest to the Chordata; therefore, their study has particular interest in the framework of the biochemistry of evolution.

Very few results have been collected on the endocrinology of the Enteropneustes, of which *Balanoglossus* is the most widely studied representative. The fixation of labeled iodides by *Dolichoglossus kowalevskii* and their localization in the epithelium of shallow in tegumentary glands, but in no other places, has been established (*257*). Analogous results have been obtained in *Glossobalanus;* chromatographic investigation of

I^{131}-labeled constituents showed that only iodides were present (*258*). The biosynthesis of thyroid hormones, then, does not take place in this member of Enteropneustes, and this, as will be seen below, is one of the indexes of "one of the most fundamental divisions in the animal kingdom" (*259*). On this level as on others, the Enteropneustes are correctly separated from the Protochordata; they correspond to an earlier stage of evolution.

Among the Urochordata (Tunicata), there are fixed forms (ascidia) and pelagic forms (*Salpa* and others); only the former have been the subject of extended studies, which dealt principally with two organs that are considered to be endocrine in nature: the subneural gland and the endostyle. The former arises from the middle portion of the larval neural tube of the ascidia and has been too hastily compared to the hypophysis of the vertebrates (*260*). It has recently been considered that this organ may not have any endocrine function (*261*), but the existence of this function remains probable though poorly defined. However, the presence of neurosecretory cells in the nerve ganglion of ascidia and in the subneural gland suggests relationships analogous to those between the hypothalamus and the hypophysis in vertebrates (*262*). With regard to certain secretory regions of the endostyle, derived from the primitive pharynx like the thyroid gland in vertebrates, it has been deemed appropriate to attribute to them the same functions as the latter, although the biosynthesis of thyroid hormones in the ascidia is not localized solely in the endostyle (*261, 263*).

Turning to the subneural gland, the secretion of chromatophoric hormone is doubtful whereas that of a product with oxytocic activity is likely (*264*). However, this product, of unknown nature, is not identical with the oxytocin of vertebrates; extracts of other tissues of the ascidia, like that of the subneural gland, possess weak oxytocic activity. The production of a gonadostimulin, a hormone causing the emission of genital products, by the ascidian subneural gland has been reported (*265*) but is still under discussion. Injection of an extract of the subneural gland causes the emission of sperm and eggs in the ascidia, but it has no effect if the nerve ganglion has been destroyed or the genital nerve severed. Therefore, it has been postulated that the neural gland secretes a gonadostimulin which acts on the nerve ganglion to release the influx which causes emission of the genital products. Although the gonadostimulin obtained from the urine of pregnant women is also active in this process, there is no chemical information to support a structural analogy between the two hormones.

The presence in the tunicates of thyroid hormones identical with those of the vertebrates, namely L-thyroxine (T_4) and 3,5,3′-triiodo-L-thyronine (T_3)is firmly established. Its significance is great in terms of the biochemistry of evolution since, contrary to a very widely held opinion (*266*), there are no thyroidal structures in invertebrates of groups below the Protochordata. As discussed in a previous chapter (*267*), the fixation of iodine by primitive invertebrates, which is widespread among sponges, certain corals, and certain mollusks, does not indicate the presence of a thyroidal structure, but rather the formation of iodinated scleroproteins (spongins, gorgonins, constituents of the fibers of the byssus, of the periostracum). Even if the latter contain traces of T_3 and T_4, they do not liberate them to put them at the disposal of cellular receptors; thus they do not meet a condition which is a prerequisite for the action of any hormone.

Iodine fixation by the tunicates has been the subject of a series of radioautographic (*261*) and biochemical (*263, 268*) studies which led to the following main results. Preliminary observations showed that I^{131} is localized in the stolonic septum of certain ascidia reproducing by budding (*269*). The systematic investigation of the distribution of I^{131} in various tissues of ascidia which reproduce by oviposition, such as *Ciona, Dendrodoa,* and *Botryllus,* has established the multiplicity of loci of concentration of I^{131}, as shown by autography (*261*) or direct measurement of radioactivity (*263*). One of these loci is in certain regions of the endostyle, but most of the I^{131} is fixed in the tissue lying below the cellulose cover of the ascidia, from which it has been possible to extract an iodoprotein which exhibits certain similarities with the thyroglobulin of vertebrates (*268*). A butanol extract of various tissues contains two thyroid hormones, T_3 and T_4, in the free state, and the iodinated protein extracted from the tunic contains these in association with 3-monoiodotyrosine and 3,5-diiodo-L-tyrosine, so that the biosynthesis of T_3 and T_4 appears to take place by the same mechanism in tunicates as in vertebrates. The extent of this biosynthetic process is relatively small in their endostyle compared with that taking place in the endostyle of cyclostome larvae, in which the thyroidal structure is localized exclusively in that organ. However, the homology of the organ designated by the same name in the two cases is very crude and leads to regrettable confusion to which numerous authors have added. The interest focused on the biosynthesis of thyroid hormones in the tunicates is actually related more to the biochemistry of evolution than to its preferential localization in a morphologically undifferentiated tissue containing migratory cells of mesenchymatous origin.

The physiological significance of the presence of T_3 and T_4 is quite obscure since the tunicates are reputed to be insensitive to T_4 and the data collected on its action on the metamorphosis of their larvae are contradictory (*270*). It is not impossible that, among the numerous stages of larval development, the "tadpole larva" of the ascidia—with an anterior portion corresponding to the pharyngeal region and a tail containing the outline of a dorsal chord—may be particularly sensitive to the metamorphogenic action. This problem will defy investigation until homogeneous cultures of larvae at a well-defined stage are achieved, whereas at present the only available cultures contain mixtures of larvae at different stages of development. Important physiological problems, then, have been opened up by the biochemistry of thyroid hormones in the tunicates (*271*).

In the Cephalochordata, the formation of the thyroid hormones and their utilization at the level of the receptors take place as in the larvae of the cyclostomes (Ammocoetes), to which *Amphioxus* exhibits pronounced similarities. The endostyle derived from the primitive pharynx is, in certain of its regions, the site of fixation of I^{131} and of the formation of the hormones (*272, 273*). These arise within a thyroglobulin, in cells which desquamate and are carried along by the pharyngeal mucus to the digestive tube, where T_3 and T_4 are liberated by proteolysis and resorbed by the intestine. They are found in the free state in tissues which fix them from the humors derived from the blood. The existence of a functional thyroidal rudiment in the adult Cephalochordata no longer poses particular problems with regard to the scheme of the biochemistry of evolution. However, this fact, in the presence of the same hormones in the Urochordata but not below them in the zoological classification, provides a significant biochemical argument for the conclusion that the tunicates constitute a fundamental stage in type differentiation. The biosynthesis of thyroid hormones, with its definite mechanism, appears in these organisms well ahead of the morphological organization of secretory formations; in this respect, biochemical evolution precedes morphological evolution. With the endostyle of the Cephalochordata, a definite stage is passed over where in the Urochordata a very imperfect rudiment is seen. Finally, since the formation of 3-monoiodo- and 3,5-diiodo-L-tyrosine in many lower invertebrates should no longer be considered to be an index of primitive thyroid function, the Urochordata constitute the first group at whose level this function appears, and it does so with the same biochemical mechanisms as those in all the vertebrates (*274*).

VI. Pheromones

The term pheromones was introduced by Karlson and Lüscher (*275, 276*) to characterize a group of substances "which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental response." The main difference from the true hormones is the action among individuals. The best example is the sex attractant of a moth, which is secreted by the female as an air-borne stimulus. It releases in the male a well-defined pattern of behavior, finally leading to copulation.

In *Bombyx*, this is a whirling dance which is easy to observe; it has been used as bioassay for the purification of the sex attractant.

A. SEX ATTRACTANTS

1. *Bombykol, the Sex Attractant of B. mori*

The isolation of this substance was possible only through large-scale extraction; 500,000 scent glands were collected and extracted with petroleum ether; the extract was fractionated by solvent extraction, countercurrent distribution, and chromatography. Finally, Butenandt *et al.* (*277*) succeeded in isolating 12 mg. of the pure, crystalline *p*-nitroazobenzenecarboxylic ester of the sex attractant. The ester is inactive, but the alcohol freed by alkaline saponification is highly active, a solution of 10^{-10} μg. per milliliter of petroleum ether corresponding to the attractant unit.

Oxidation of the pure ester with $KMnO_4$ yielded three fragments: butyric acid, oxalic acid, and ω-hydroxydecenoic acid in form of the *p*-nitroazobenzoic derivative. Therefore, the structure of $\Delta^{10,12}$-hexadecadienol for the active principle could be deduced (*278*):

$$H_3C—CH_2—CH_2—CH=CH—CH=CH(CH_2)_8—CH_2OH$$

Synthesis revealed that the natural compound was the 10-*trans*-12-*cis* isomer. The name "bombykol" has been given to this sex attractant.

The response of the *Bombyx* male is highly specific in respect to the structure. The geometrical isomers which have also been synthesized are virtually inactive, they are even inhibitory: in mixture with bombykol, they depress the activity so that 10^{-3} to 10^{-5} μg. per milliliter of bombykol are necessary to give the activity of one unit (*279, 280*).

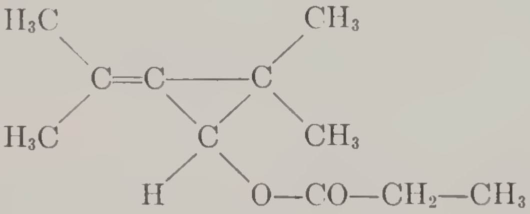
The electrophysiology of the response of the *Bombyx* female has been investigated (*281, 282*). With microelectrodes, slow changes in the potential of the antennae have been demonstrated; this "electroantennagram" is characteristic for the olfactory response. It can be calculated that only few molecules—perhaps a single molecule—suffice to excite the receptor cell and that the behavioral response is presumably released by the signals of a few chemoreceptors.

2. *Gyptol*

Another lepidopteran attractant has been isolated from the female gypsy moth (*Porthetria dispar*) shortly later by Jacobson *et al.* (*283,* *284*). The substance, named gyptol, has been tentatively identified as 10-acetoxy-7-cis-hexadecenol-1, and the synthesis of the active substance has been reported (*284a*). The compounds prepared by Jacobson have been assayed with electrophysiological methods; the D- and L-compounds are equally active in producing the characteristic "antennogramm" (D. Schneider, personal communication, 1963).

3. *Other Sex Attractants*

Some other sex pheromones have been investigated in some detail. In the American cockroach, *Periplaneta americana,* the female produces an attractant which acts on the male as excitant and releaser for copulation behavior. The substance has been extracted (*285, 286*) and isolated in pure form (*286a*). The accompanying formula (II) has been assigned to the *Periplaneta* sex attractant (*286b*).



(II)

In a tropical water bug, *Belostoma indica,* the males contain special secretory ducts that contain a substance with a strong odor. The substance has been isolated and identified as Δ^2-hexenol acetate (*287*):

$$H_3C—CH_2—CH_2—CH{=}CH—CH_2O—CO—CH_3$$

The substance has been synthesized, but biological experiments on its role in *Belostoma* have not been made. A very similar compound, Δ^2-hexenal, has been detected in both sexes in the cockroach *Eurycotis* (*288*).

B. Pheromones of Social Insects

In social insects, communication between the members of the group is of special importance. Numerous phenomena have been described that may involve pheromones. Only some well-studied examples will be discussed here; for further information the reader is referred to recent reviews (*276, 289, 289a*).

Ants and termites often travel on trails which are marked by some chemical special secretions. In the case of *Iridomyrmex detectus,* the active principle has been identified as Δ^2-metylheptenone (*290*). For other trail substances of ants (*291, 292*) and termites (*293*), chemical data are not yet available; however, Δ^3-hexenol-1 (the *cis* compound) has been found to act as trail substance (*293a*). It has been isolated from nest material, though it is uncertain if it is a termite product.

The trail pheromone of the honey bee, produced by the Nassanoff gland (*294*), has been tentatively identified as geraniol (*294a*). The sting of the bee carries another pheromone releasing alarm and attack behavior. It seems to be a mixture, containing as one component isoamyl acetate (*294b*).

One other pheromone of the honey bee, the so-called "queen substance," has recently been isolated and identified by two groups of workers (*295–297*) as an unsaturated keto acid:

$$H_3C{-}CO{-}(CH_2)_5{-}CH{=}CH{-}COOH$$

The biological function of the queen substance is to inform the members of the colony, i.e., the worker bees, of the presence of the queen. The substance is present on the cuticle of the queen; on withdrawal of the queen, i.e., the queen substance, new queen cells are constructed and some of the larvae are raised as queens; also, ovary development in the workers is no longer inhibited.

A similar phenomenon is encountered in termite colonies, although the production of new functional kings and queens is brought about by other means. Through an induced molt, the workers become adult and "supernumerary reproductives." For details, the reader is referred to the publications of M. Lüscher (*239, 241, 298, 299*).

The queen substance has nothing to do with the royal jelly. The latter is the food of larvae which are destined to become queens. It is still an open question whether this food contains a certain factor responsible for the development of queen characters or whether only the large supply of high-quality food, containing fairly large amounts of proteins, vitamins, and other special constituents (*300–303*), is responsible.

VII. Concluding Remarks

Despite the paucity of precise data on numerous hormones of the invertebrates, which have merely been shown to be active, certain conclusions become apparent.

The chemical mediators of nerve transmission, such as acetylcholine, adrenaline and noradrenaline, and 5-hydroxytryptamine, are very widely distributed in invertebrates as well as vertebrates. Although sometimes discovered in vertebrates, e.g., 5-hydroxytryptamine, their presence in invertebrates indicates the existence of very general, and therefore not specific, physiological mechanisms; other particular neurohumors may doubtless be associated with them, such as hydroxytyramine in insects or urocanylcholine in mollusks.

There is as yet no chemical basis for discussion of the products of neurosecretion; the evidence available consists of physiological analogies between active extracts of invertebrate or vertebrate organs and of histochemical data, since the investigator is frequently reduced to record the existence of a secretion the nature of which remains to be established. The same is true of the many hormones apart from the products of neurosecretion. There are a very few exceptions among the insects and tunicates: they illustrate two pathways to obtaining data on the comparative biochemistry of the invertebrates.

In insects, the demonstration of hormones particular to a zoological group indicates the biochemical specificity of its endocrine mechanisms. It is probable that the hormones of the subneural gland of the tunicates occupies an intermediate position in this regard, since the administration of certain vertebrate hormones can cause some of their effects. On the other hand, the fact that thyroid hormones identical to those of vertebrates are secreted by Protochordata, but not by the invertebrates situated below them in zoological classification, poses a problem of a different sort.

The appearance of an endocrine function during evolution has a very significant biochemical aspect, since it is essential to establish whether or not the secreted hormone is identical to that found at higher stages of evolution. On the one hand, the same biological activity may be vested in substances of different constitution, one of which would be the precursor, the physiological rudiment of the other during phylogenesis. On the other hand, the creation of an endocrine mechanism may rest on the biosynthesis of a product whose structure is somehow fixed from a certain zoological group on; it then signifies a genuine biochemical differentiation at the stage of evolution where it sets in. The most noteworthy example, without doubt, is the appearance in the tunicates of the thyroid hormones, which are found with unfailing phylogenetic continuity up to the most highly developed vertebrates, whereas they are lacking in the invertebrates below the tunicates.

A similar situation may be encountered in the phylum Arthropods. It has been pointed out that the molting hormone, ecdysone, is active not only in all orders of insects studied, but also in the crustaceans. Hence, the basic mechanism of hormonal control of molting may have been acquired early in the phylogeny and later adapted to the special needs of the species. Hemimetaboly and holometaboly may be regarded as specializations of the indefinite molts in the lower crustaceans and thysanurans.

From the chemical point of view, it is most interesting that ecdysone, like many vertebrate hormones, belongs to the class of steroids. It is tempting to speculate that the steroid nucleus has some inherent property especially suitable for a certain mechanism of action—perhaps an interaction with the genetic material (*304*).

In the lower phyla of invertebrates, one finds only neurohumors, chemical mediators with almost universal mechanisms. A first level of complexity is represented by the specialization of certain nerve cells for the elaboration of substances that act directly on specific receptors. An example is the inhibitory principle of epitoky in polychete annelids. At a higher level of complexity, the substance produced by the specialized neurons no longer acts directly on the hormonal receptor but on an endocrine gland which does not have an ontogenetic relation with the nervous system; it is then the latter gland which elaborates the hormone acting on the receptor. This state of affairs is present in the arthropods. In fact, the control of molting in insects and crustaceans is accomplished by the secretion of a stimulin of cerebral origin; this stimulin activates the endocrine gland responsible for the secretion of ecdysone, which acts on the receptors. Thus, a striking resemblance exists here with the hypothalamopituitary complex of the vertebrates, despite a wide difference between the two neuroendocrine systems with regard to their functional significance. All indications are that the tendency which manifests itself during evolution is one of progressive increase in complexity of humoral mechanisms, with multiplication of the hormones participating in the regulation of a physiological phenomenon and multiplication of the organs which produce those substances. The resemblances which are called forth may be the result of a convergence, not that of an orthogenesis.

Development of a highly complicated system of hormone-like mechanism is also encountered in the group of pheromones. Pheromone-like substances are presumably to be found in all phyla, but the most elaborate mechanisms of this kind have been revealed among the insects, especially the social insects. Apparently, they developed during acquisition of social life by the bees and termites.

The comparative biochemistry of the hormones, then, presents tremendous possibilities for research on the invertebrates. Its approaches are multiple ones, and many of its problems are already accessible to biochemists, thanks to the knowledge which has been gathered concerning their physiological and morphological aspects.

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CHAPTER 6

Protein Hormones in Vertebrates*

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I. Introduction . . . 299
  A. General Considerations on Hormones . . . 299
  B. Chemical Classification of Protein Hormones . . . 300
II. Group of the Neurohypophyseal Hormones . . . 302
  A. Biological Properties . . . 302
  B. Purification . . . 304
  C. Chemistry . . . 308
  D. Evolution in the Domain of the Neurohypophyseal Hormones . . . 313
III. Group of the Melanocorticotropic Hormones . . . 316
  A. Biological Properties . . . 317
  B. Purification . . . 318
  C. Chemistry . . . 321
  D. Relationships between Biological Activities and Structure . . . 327
IV. Group of the Insulins . . . 330
  A. Biological Properties . . . 331
  B. Purification . . . 332
  C. Chemistry . . . 333
  D. Relations between Biological Activity and Structure . . . 336
V. Conclusions . . . 339
  References . . . 340

I. Introduction

A. General Considerations on Hormones

In the invertebrates, it has not yet been possible to relate most hormonal actions to definite structures; their study, therefore, remains almost entirely at the physiological level (as shown in Chapter 5 of this volume, by Gabe *et al.*). In the vertebrates, by contrast, a relatively large number of hormones of various types have been isolated, which are secreted specifically by each of the endocrine glands; thus, it has become feasible to attempt a study of their comparative biochemistry. The processes which involve them are at the same time very diverse and peculiar to the different active structural types.

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* Translated from the French by Dr. Erich Hirschberg, College of Physicians and Surgeons, Columbia University, New York.

Each group of hormones of relatively simple constitution, such as those of the adrenal cortex or medulla, of the thyroid, or of the gonads, exhibits the same structure in all vertebrates. However, certain derivatives of only slight physiological interest—e.g., equilenin and equilin among the estrogens—are secreted in particularly large quantities by a particular species. Further, quantitative differences occur in metabolic pathways, such as the predominance of sulfoconjugation of a hydroxylated steroid or an iodothyronine in certain species and of glycuronconjugation in others; but no general concepts can as yet be derived from these isolated facts, so that it would be of little interest to draw up an inventory of these.

The study of the protein hormones of the vertebrates, on the other hand, is already accessible to comparative biochemistry, since the existence of specific differences in their structure has been established by an impressive number of investigations. During the last twenty years, in fact, protein hormones of several groups have been isolated in the pure state from the endocrine glands of various animals, belonging in some cases to classes far removed from each other. The structure of some of them has been established, and peculiarities pertaining to each species have been demonstrated. This is true for the neurohypophyseal hormones, the melanocorticotropic hormones, the insulins. Progress during the last ten years has been so great that today some of these substances are among the best-known proteins or peptides of high molecular weight; therefore, it now seems appropriate to attempt to present their comparative biochemistry, which can be based on firm and very extensive chemical notions. This is the objective of this chapter, intentionally limited to those hormones of the vertebrates that exhibit, as a function of their origin, structural peculiarities which can be related to differences in biological action. Each section will deal independently with the study of a group of hormones, since it did not appear possible to engage in a discussion of general character without being able to extend legitimately, from one group of compounds to the next, observations made only on one of them. The present state of research in this field has made it possible, in each instance, to relate the activity of the protein hormones to certain elements of their structure, even though the phylogenetic filiation of representatives of the same group during evolution remains imprecise.

B. Chemical Classification of Protein Hormones

The notion of hormonal action consists of two elements: on the one hand, the "chemical message" secreted by the endocrine gland and transported by the blood stream; on the other hand, the receptor cell. The adaptation of one to the other in a given species has not been accomplished right off. It is conceivable that agent and receptor, each under the control of a particular genetic system, may be modified autonomously during evolution of the organisms and that to molecules formed a long time ago there may correspond, in more recent species, new receptors; certain molecules may even subsist as fossils without any particular function. For example, arginine-vasotocin, which has recently been isolated in fish, batrachians, and birds, probably does not play the same biological role in the different classes of vertebrates; the intermedins, whose action on the melanocytes of the frog is so spectacular, do not appear to have any biological significance in mammals, where, nonetheless, they are present in very appreciable quantities. Moreover, the determination of the structure of several polypeptide hormones has, in some instances, shown a direct chemical relationship between molecules having different biological properties, such as the oxytocins and vasopressins; it sometimes appears convenient to bring together in the same chemical group active principles having very different physiological actions. But when the attempt is made to classify these protein hormones on an exclusively structural basis, the limits of the chemical family become very difficult to define. Should we consider as part of the same family those hormones which have in common an amino acid sequence of sufficient length so that this arrangement cannot be due to chance? Or should we limit ourselves to the molecules which have, in addition, the same number of constituent amino acids? In the hog pituitary, for example, there are three peptide hormones, each composed of a different number of amino acids but obviously related since the structural analogy extends to eleven residues: by reason of their biological properties, they are called α-melanocyte-stimulating hormone (α-MSH), β-melanocyte-stimulating hormone (β-MSH), and corticotropin. It is possible that these peptides are all derived from a common original molecule by a mechanism of duplication followed by differentiation, and in this connection it would be interesting to investigate whether this single ancestral molecule is present in the most primitive vertebrates. Be that as it may, it has been possible also in other mammalian species to isolate three hormones resembling, respectively, hog α-MSH, β-MSH, and corticotropin, in particular in the number of residues. Therefore, it seems necessary to postulate the existence of three distinct hormonal families belonging to the same chemical group, without presupposing the biological role of each of them. Similarly, among the neurohypophyseal hormones we can distinguish a family of oxytocins and a family of vasopressins as part of the same structural group. This review of the comparative biochemistry of the protein hormones will arbitrarily be limited to the hormones whose structure is fairly well known in several species of vertebrates.

II. Group of the Neurohypophyseal Hormones

This group includes several hormonal families that have a common structural feature on the chemical level which on the biological level is expressed by common activities, although the degree of each activity varies greatly from one hormone to the next. The homogeneity of the group makes it possible to study together the biological properties, then the purification procedures. A special section (II, C) will be allocated to the oxytocins, the vasopressins, and the vasotocins isolated up to the present. Finally, the distribution of the hormones in the vertebrate scale will be considered in relation to evolution.

A. Biological Properties

The isolation of several hormones in the pure state has made it possible to establish that each of them possesses intrinsically several biological activities, some considered to be pharmacological, the others probably physiological. Those which lend themselves to rapid and precise assay have received particular attention from the chemists, who consider them as specific reagents for the determination of the hormone content of the preparations and, consequently, for guidance in the purification of the active principles. Two activities, the oxytocic and vasopressor, have been used so extensively for this purpose that they have given their name to hormones even though the physiological action of the hormones in the body may be different. Nevertheless, these two tests, which require mammalian organs, have permitted the discovery and purification of substances present in the lower vertebrates, the study of the biosynthesis and degradation of the hormones, and the determination of the importance of constituents by comparison of the natural products and synthetic analogs; in this way, they have been extremely valuable.

In general, it is best to make all estimations of activity with reference to an international standard and with the consistent use of a given organ of the same species. In fact, the uterus of the hen and the uterus of the rat react differently according to the structure of the hormone acting on them. The international standard used is an acetone powder of beef posterior pituitary which by convention has 2 units per milligram. Detailed reviews have been devoted to the physiology and pharmacology of the neurohypophyseal hormones (*1–5*).

1. *Action in Mammals*

a. Oxytocic Action. This is the contracting action on the uterine muscle. The uterus of the guinea pig or of the cat is sometimes employed, but the most generally used test object for the determination of this activity is the uterus of the rat (*6*). The isolated organ is immersed in a small jar containing a physiological solution of appropriate composition, maintained at 32°. The addition of hormone to the solution causes contraction of the muscle, which moves a lever that records the contractions with amplification on a kymograph. The concentration of magnesium used in the physiological solution modifies the response of the organ (*7*).

b. Vasopressor Activity. This is the increase in blood pressure which is observed in the anesthesized mammal. Generally, this action is measured in the rat; the hormone is injected into the leg vein, and the increase in pressure is determined in the carotid artery (*8*).

c. Milk-Ejecting Activity. In the lactating female, the injection of hormone causes the ejection of the milk following the contraction of the mammary muscle. The assay is carried out in the rabbit: the hormone is injected into the ear vein, and the variations in pressure inside the mammary gland are measured with a manometer connected to one of the nipples (*9*).

d. Antidiuretic Activity. In the hydrated animal, the hormone causes a diminution in the urinary flow. This action is usually measured in the rat (*10*) or dog (*3*) after intravenous injection of the active principle. The effect of the various hormones has also been studied in man (*11*).

2. *Action in Birds*

a. Depressor Activity. The injection of hormones in the anesthetized chick causes a lowering of the blood pressure (*12*). The hormone is injected in the femoral vein, and the pressure is measured in the ischiatic artery.

b. Oxytocic and Antidiuretic Activities. Oxytocic and antidiuretic actions have been observed in the chick (*13*), but these properties have not yet been used for systematic assays.

3. *Action in Reptiles*

Very little is known about the action of neurohypophyseal hormones in reptiles. An oxytocic action has been demonstrated in the turtle (*13*) and an antidiuretic effect in the crocodile (*14*).

4. *Action in Amphibians*

a. Hydroosmotic Activity. This is the effect on water transport across the bladder wall in the frog (*7*) or the toad (*15*). The movement of the water depends on the osmotic gradient; the hormone acts only to increase the permeability. This permeability-increasing effect is also seen *in vivo* on the skin of the frog (*16*).

b. Natriferic Activity. This is the action on the active transport of sodium across the surviving frog skin. In the absence of an osmotic gradient, there is seen across the skin a net sodium flux whose intensity is measured by the current which goes across the skin (*17, 18*).

c. Antidiuretic Activity. An antidiuretic activity of certain neurohypophyseal hormones has been shown in the batrachians (*19, 20*).

5. *Action in Fish*

a. Spasmodic Activity. In fish, the only biological effect of the neurohypophyseal hormones which is unquestioned is a spasmodic action in connection with the emission of sexual products (*21*). The intravenous injection of the hormones is made in *Fundulus;* the unit of activity is the smallest dose causing a spasm during the first 40 minutes after the injection.

Table I shows the values of the different biological activities for each of the four neurohypophyseal hormones isolated up to the present.

B. Purification

The purification of the neurohypophyseal hormones of mammals, birds, batrachians, and fish was undertaken, and it has proved possible to obtain pure substances from each class of vertebrates.

1. *Hormones of Mammals*

Taken up at the turn of the century, the purification of the neurohypophyseal hormones of beef was not achieved until about fifty years later, when new techniques, e.g., countercurrent distribution or ion-exchange chromatography, could be applied. Du Vigneaud and his collaborators used mostly countercurrent distribution to obtain oxytocin and vasopressin in a high state of purity. After separation of the two hormones by fractional precipitation with ether and hexane, oxytocin is purified by two successive distributions in the system secondary butanol-0.05% acetic acid ($K = 0.4$) (*22*) and vasopressin by two distributions in the system normal butanol-0.09 M p-toluenesulfonic acid ($K = 1.25$) (*23*). The American authors succeeded in obtaining oxytocin crystallized as the flavianate, but vasopressin has not yet been crystallized. Hog oxytocin and vasopressin have been isolated by means of the same methods (*22, 24*).

Fromageot and his group also succeeded in purifying the two hormones by adsorption on silica and, after separate elution, by submitting oxytocin to a distribution in the system secondary butanol-0.5% acetic acid (*25*) and vasopressin to a distribution in the system normal butanol-salicylic acid-water (*26*). Then investigators turned to chroma-

TABLE I

Biological Activities of the Neurohypophyseal Hormones[a]

Hormone	Tests in mammals				Tests in birds		Tests in batrachians	
	Oxytocic activity (rat)	Pressor activity (rat)	Milk-ejecting activity (rabbit)	Antidiuretic activity (dog)	Oxytocic activity (hen)	Depressor activity (fowl)	Hydroosmotic activity (frog)	Sodium-transferring activity (frog)
Oxytocin (mammals)	500	7	500	3	35	500	500	500
Lysine-vasopressin (hog, hippopotamus)	4	300	66	45	23	48	13.5	8
Arginine-vasopressin (mammals)	50	600	100	600	480	85	80	20
Arginine-vasotocin (fowl, frog, bony fish)	75	125	155	100	1280	150	14,000	1100

[a] The values (in U.S.P. units per milligram) shown for oxytocin, lysine-vasopressin, and arginine-vasopressin are for natural products; they are calculated according to van Dyke and collaborators (*4*, *13*) for the tests in mammals and birds and according to Jard *et al.* (*20*) for the tests in batrachians. The values shown for arginine-vasotocin are for a synthetic product; they are calculated according to Katsoyannis and du Vigneaud (*61*) and Munsick *et al.* (*13*) for the tests in mammals and birds and according to Jard *et al.* (*20*) for the tests in batrachians.

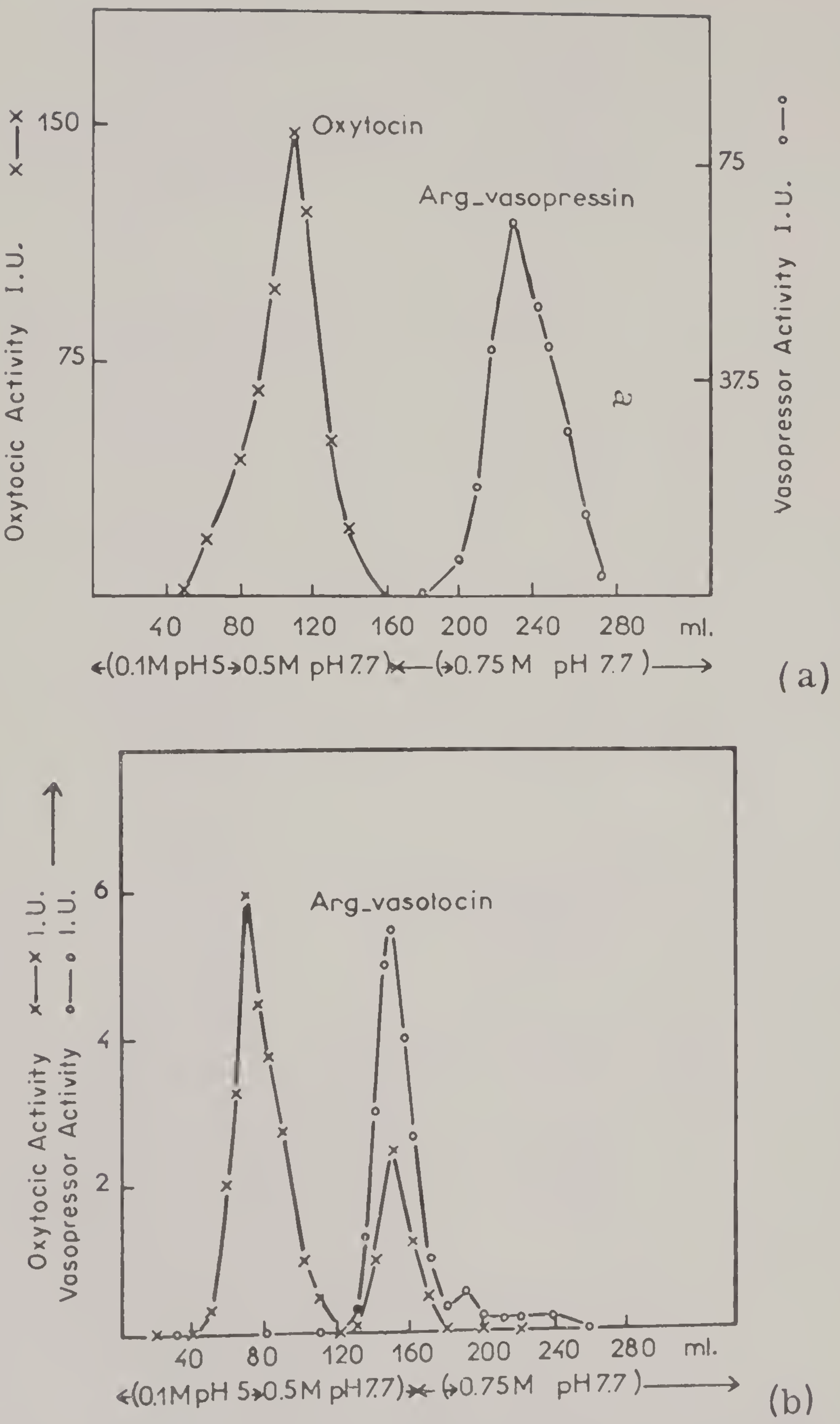

Fig. 1. Chromatography of neurohypophyseal hormones on Amberlite IRC-50. the hormones are adsorbed at the top of a resin column (0.9 × 10 cm.) form H; the column is then equilibrated with 0.1 *M* ammonium acetate buffer at pH 5.0 and the gradient is successively made with 0.5 *M* and 0.75 *M* solutions at pH 7.7. (a) Mammal: sheep. (b) Fish: pout (*Gadus luscus*).

tography. Porath (*27*) used trimethylaminoethylcellulose whereas Schally *et al.* (*28*) employed carboxymethylcellulose. However, the recommended procedures are laborious because they require several chromatographic runs and the purity of the products as well as the yields have not been controlled rigorously. A method for the simultaneous purification of the two hormones has been proposed by Acher *et al.* (*29*) which has made it possible to isolate the active principles of several species of mammals. The method utilizes the fact that both oxytocin and vasopressin are associated in a stable complex with a protein of the posterior pituitary, *neurophysin.* In a first step, the neurophysin-hormones complex is precipitated with sodium chloride at pH 3.9 from an extract of the glands. In a second step, the protein carrier is eliminated by precipitation with trichloroacetic acid. In a third step, the hormones are separated by chromatography on Amberlite IRC-50, using a pH and ionie strength gradient (Fig. 1). This procedure has led to the isolation of the hormones of man (*30*), horse (*31*), sheep (*32*), and whale (*33*). At present, oxytocin and vasopressin have been identified in six species of mammals. In all instances, the oxytocin found is the same; the vasopressin is the same in five species, but the hormone of hog is slightly different from that found generally in that a lysine residue replaces an arginine residue (lysine-vasopressin in place of arginine-vasopressin). According to the pharmacological data of van Dyke and collaborators (*34, 35*), arginine-vasopressin is present in the monkey, the dog, the rat, and the camel as well as in the nonplacental mammals.

2. *Hormones of Birds*

The success encountered in the purification of neurohypophyseal hormones in mammals stimulated analogous work in birds. Here the initial quantities of material are obviously much smaller and only a purification procedure with a high yield can be used. The fowl (*Gallus domesticus*) was chosen since it was possible to obtain a large number of glands of this animal. Furthermore, the posterior pituitary is easily separated from the anterior pituitary. About 15,000 glands yielded 6 gm. of posterior pituitary acetone powder which assayed at approximately 0.4 U.S.P. units of oxytocic and vasopressor activities. With these amounts, it has been possible to obtain three preparations of hormones. To increase the yield, neurophysin of exogenous origin was added to the extracts, but the procedure in its broad outlines was identical to that used in the case of the mammals. Two hormones could be characterized: oxytocin and arginine^8-oxytocin or arginine-vasotocin (*36, 37*).

3. *Hormones of Batrachians*

The frog (*Rana esculenta*) was chosen because of its alimentary use in France and the possibility of separating the two lobes of the hypophysis. Twenty thousand glands yielded 2 gm. of a posterior pituitary acetone powder with an activity of approximately 1 U.S.P. unit of oxytocic and vasopressor activities. The procedure employed was identical with that chosen to purify the hormones of the chicken. Until now, only one hormone has been characterized: arginine^8-oxytocin (*38*). Another hormone with oxytocic activity has not yet been identified.

4. *Hormones of Fish*

In fish, it is not possible to separate the anterior and posterior lobes of the pituitary, which are profoundly connected. It is therefore necessary to use the entire gland. Purification has been undertaken in three species of marine bony fish, all belonging to the family of the Gadidae. They are the pout (*Gadus luscus* L.) (*39*), the pollack (*Pollachius virens* L.) (*40*), and the hake (*Merluccius merluccius*) (*41, 42*). In the case of the pout, 10,000 glands yielded 3.7 gm. of pituitary acetone powder assaying at 0.5 U.S.P. units of oxytocic and vasopressor activity. The purification procedure is essentially the same as that adopted for the hormones of the other vertebrates (*39*). In the second instance, 125 gm. of pituitary acetone powder of pollack were used in the same procedure (*40*). In the third instance, countercurrent distribution was applied to a fraction of a pituitary extract of hake (*42*). With regard to the pout, a hormone, arginine^8-oxytocin, has been identified by its amino acid composition and by a study of its structure. In the pollack and the hake also, a peptide is found which has the amino acid composition and the biological properties of arginine^8-oxytocin (*40, 42*). Another hormone, with oxytocic activity, has been demonstrated in these species but not yet identified.*

C. CHEMISTRY

Up to this time, four neurohypophyseal hormones have been isolated and characterized in the vertebrates. The chemical structures of these four principles, which have been proved by synthesis, are very similar. In each instance, they are peptide molecules composed of a chain of nine amino acid residues. The differences between the hormones consist of a change in the residues in position 3 or 8 of the chain. Since the biological activities do not seem to be specific, it appears preferable to adopt names based on the structure. Oxytocin was the first neurohypophyseal hormone characterized, and we have proposed to consider the other hormones as natural analogs derived by the substitution of certain residues, with a prefix indicating the nature and position of the substituted residues (*43*). In this nomenclature, the four known hormones are oxytocin, arginine^8-oxytocin, phenylalanine^3-arginine^8-oxytocin, and phenylalanine^3-lysine^8-oxytocin.

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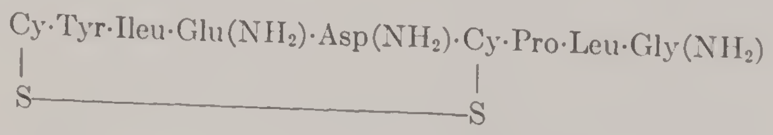
* *Note in proof:* This "oxytocin-like" hormone, called isotocin, has been isolated and seems to be the Ser^4-Ileu^8-oxytocin.

1. *Oxytocin*

a. Structure. The quantitative amino acid composition of oxytocin was established by du Vigneaud and his collaborators (*22*) with the use of starch column chromatography. The molecule contains eight amino acids in stoichiometric quantities: cystine, tyrosine, isoleucine, glutamic acid, aspartic acid, proline, leucine, and glycine. After hydrolysis, there are three molecules of ammonia, derived from the three amide groups. The calculated molecular weight is 1007.

The structure of the hormone was established independently by du Vigneaud *et al.* (*44*) and by Tuppy and Michl (*45*). The American investigators found at first that the peptide had only one *N*-terminal end belonging to cystine. Applying to oxidized oxytocin the technique of recurrent degradation of Edman, they determined the sequence of the first four residues. They then deduced the rest of the sequence by isolating by column chromatography the short peptides produced by partial acid hydrolysis and then characterizing them. The *C*-terminal end as well as the β- and γ-carboxyl groups of the aspartic and glutamic acids are amided. These results agree with the value for the isoelectric point of oxytocin (i.p. = 7.7), the peptide having a free amino group but no free carboxyl group. Finally, the position of the intrachain disulfide bridge was determined by the localization of the two cysteic acid residues resulting from the oxidation of the cystine.

Tuppy and Michl (*45*) studied the peptides produced by partial acid and enzymatic (subtilisin) hydrolysis of oxidized oxytocin. The peptides were fractionated by electrodialysis and purified by paper chromatography. The characterization of the different fragments permitted the determination of the complete sequence of oxytocin. The isolation of glycinamide from the enzymatic hydrolyzate revealed the *C*-terminal residue and also provided direct proof for the presence of an amided *C*-terminal end. The concordant results obtained by the two groups of investigators make it possible to assign the following structure to beef oxytocin:



$$\underbrace{\mathrm{Cy}\cdot\mathrm{Tyr}\cdot\mathrm{Ileu}\cdot\mathrm{Glu(NH_2)}\cdot\mathrm{Asp(NH_2)}\cdot\mathrm{Cy}}_{\mathrm{S}\text{———}\mathrm{S}}\cdot\mathrm{Pro}\cdot\mathrm{Leu}\cdot\mathrm{Gly(NH_2)}$$

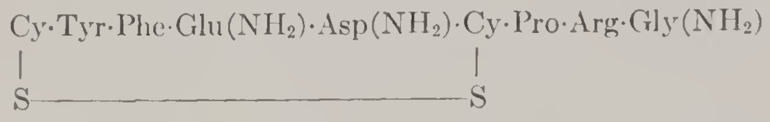
The oxytocins of other mammalian species—hog (*22*), man (*30*), horse

(*31*), sheep (*32*)—have also been isolated. Their amino acid composition and, in certain instances, their structure have been determined. It appears that the same oxytocin is present in all the species studied.

b. Synthesis. The Cornell group first accomplished the synthesis of two protected cysteine peptides, one *N*-terminal, i.e., *N*-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine (*46*), the other *C*-terminal, i.e., S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (*47*); then they carried out the synthesis of the central peptide, i.e., tosyl-L-isoleucyl-L-glutaminyl-L-aspargine (*48*). The protected central and *C*-terminal peptides were condensed with the use of tetraethyl pyrophosphite; then, after elimination of the tosyl and benzyl radicals by sodium in liquid ammonia and rebenzylation of cysteine, the heptapeptide was condensed with the *N*-terminal peptide to yield the nonapeptide. The carboxyl and benzyl radicals were then eliminated by reduction with sodium in liquid ammonia; thereafter, the oxidation of the sulfhydryl nonapeptide yielded oxytocin (*49*). The end product was purified by countercurrent distribution. The synthetic material appears identical with the natural substance in its biological properties (degree of oxytocic, pressor, and milk-ejecting activities), its physical properties (melting point, specific rotation, partition coefficient, infrared spectrum, electrophoretic mobility) and its chemical properties (amino acid composition, susceptibility to reagents and enzymes). Boissonnas *et al.* (*50*) in Switzerland, Rudinger *et al.* (*51*) in Czechoslovakia, and Velluz *et al.* (*52*) in France later proposed different schemes leading to the synthesis of the hormone. Recently, Bodanszky and du Vigneaud (*53*) applied a procedure of recurrent synthesis, using the *p*-nitrophenyl esters of the amino acids: the residues were joined one by one, starting at the *C*-terminal end, with a yield of 90% and with the avoidance of racemization.

2. *Phe^3-Arg^8-Oxytocin* (*Arginine-vasopressin*)

a. Structure. The quantitative amino acid composition indicated the presence of eight amino acids: cystine, tyrosine, phenylalanine, glutamic acid, aspartic acid, proline, arginine, and glycine. The structure of beef vasopressin was determined independently by du Vigneaud *et al.* (*54*) and by Acher and Chauvet (*55*). The application to oxidized vasopressin of the recurrent degradation of Edman, the isolation of the peptides produced by partial acid hydrolysis, and finally trypsin or papain hydrolysis enabled the Cornell group to establish the complete amino acid sequence. Acher and Chauvet (*55*), for their part, determined an *N*-terminal sequence of four residues in the oxidized hormone with the aid of the techniques of Sanger and Edman, and a *C*-terminal sequence of two residues was deduced from the effect of trypsin, which hydrolyzes an arginyl-glycinamide linkage. The central portion of the sequence was established by characterizing the fragments produced by partial acid hydrolysis. The presence of three molecules of ammonia per mole of peptide indicated that the aspartic and glutamic acid residues are amided, an observation which agrees with the isoelectric point of beef vasopressin (i.p. = 10.9). Finally, the position of the interchain disulfide bridge was determined by the localization of the two cysteic acid residues resulting from the oxidation of the cystine. These results lead to the assignment of the following structure to beef vasopressin:



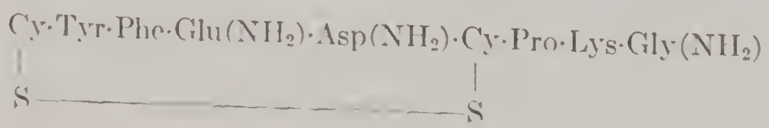
$$\mathrm{Cy\cdot Tyr\cdot Phe\cdot Glu(NH_2)\cdot Asp(NH_2)\cdot Cy\cdot Pro\cdot Arg\cdot Gly(NH_2)}$$

The study of the structure of horse (*31*) and sheep (*32*) vasopressin showed that this vasopressin (arginine-vasopressin) is also present in these species. The vasopressin of man has the same amino acid composition as beef vasopressin (*30*).

b. Synthesis. The synthesis of arginine-vasopressin was carried out by du Vigneaud and collaborators (*56*). Arginine-vasopressin was formed by coupling the azide of the *N*-terminal tripeptide, *S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine, with the *C*-terminal hexapeptide, L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginyl glycinamide. The protecting groups were eliminated by treatment with sodium in liquid ammonia and the peptide was oxidized by bubbling through air. The product was then purified by countercurrent distribution followed by zone electrophoresis. The synthetic hormone appears identical with the natural hormone with regard to its pressor activity, partition coefficient, electrophoretic mobility, amino acid composition, and chromatographic behavior on Amberlite IRC-50 (*56*).

3. *Phe^3-Lys^8-Oxytocin (Lysine-vasopressin)*

a. Structure. The amino acid composition of hog vasopressin showed this vasopressin to be different from that found until then in other mammals. Popenoe *et al.* (*24*) isolated hog vasopressin and showed that it is composed of the same amino acids as beef vasopressin except that a lysine residue replaces the arginine residue. These authors called this hormone lysine-vasopressin. Its structure was not established by degradation of the natural product, but by comparison with the synthetic product, Phe^3-Lys^8-oxytocin. The formula for the hormone is:



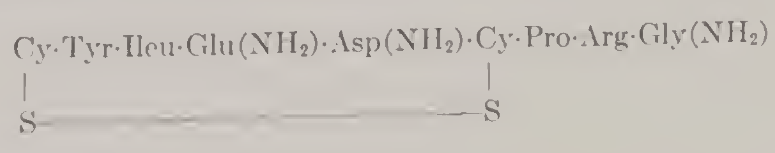
$$\mathrm{Cy\cdot Tyr\cdot Phe\cdot Glu(NH_2)\cdot Asp(NH_2)\cdot Cy\cdot Pro\cdot Lys\cdot Gly(NH_2)}$$

According to pharmacological data, lysine-vasopressin is also present in the hippopotamus, which like the hog belongs to the porcine family (*57*).

b. Synthesis. The synthesis of Phe^3-Lys^8-oxytocin was accomplished by du Vigneaud *et al.* (*58*). Lysine-vasopressin was formed by coupling the *N*-terminal pentapeptide, S-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine with the *C*-terminal tetrapeptide, S-benzyl-L-cysteinyl-L-prolyl-*N*-ε-tosyl-L-lysyl glycinamide, in the presence of *N,N′*-dicyclohexylcarbodiimide. The protecting groups of the nonapeptide were eliminated by treatment with sodium in liquid ammonia, and the peptide was then oxidized. The hormone was finally purified by countercurrent distribution and zone electrophoresis. The biological, physical, and chemical properties of the synthetic and natural products are identical (*58*). Boissonnas and Huguenin (*59*) recently obtained lysine-vasopressin by coupling the azide of the *N*-terminal tripeptide with the *C*-terminal hexapeptide.

4. *Arg^8-Oxytocin (Arginine-vasotocin)*

a. Structure. Arg^8-oxytocin was first isolated from fowl glands (*37*). The hormone was then obtained from the frog pituitary (*38*) and three marine bony fish (*39, 40, 42*) belonging to the family of the Gadidae. Its amino acid composition is similar to that of oxytocin, but the leucine residue is absent and an arginine residue is present. The molecule contains the following amino acids: cystine, tyrosine, isoleucine, glutamic acid, aspartic acid, proline, arginine, and glycine. The structure of the hormone was determined with the aid of enzymes (*60*). The *N*-terminal end was studied by exposing the preparation to leucine aminopeptidase. The enzyme liberated tyrosine and isoleucine, the *N*-terminal residue of demi-cystine remaining attached by the disulfide bridge. The same result is obtained when the enzyme acts on oxytocin, in which tyrosine and isoleucine occupy, respectively, positions 2 and 3; in contrast, when leucine aminopeptidase acts on arginine-vasopressin, one observes the liberation of tyrosine and phenylalanine, which in this molecule occupy positions 2 and 3. The *C*-terminal end was determined by allowing trypsin to act: this action results in the liberation of glycinamide, which indicates a *C*-terminal sequence of Arg·Gly(NH$_2$). A similar result is obtained when the enzyme acts on arginine-vasopressin, but no cleavage occurs when the enzyme acts on oxytocin. These findings, as well as comparison with the synthetic product, lead to the assignment of the following structure to the hormone:



$$\underbrace{\text{Cy}\cdot\text{Tyr}\cdot\text{Ileu}\cdot\text{Glu(NH}_2)\cdot\text{Asp(NH}_2)\cdot\text{Cy}}_{\text{S}\text{---}\text{S}}\cdot\text{Pro}\cdot\text{Arg}\cdot\text{Gly(NH}_2)$$

b. Synthesis. The synthesis of Arg^8-oxytocin was accomplished before the hormone was isolated. Du Vigneaud and collaborators synthesized several analogs of oxytocin and arginine-vasopressin and assigned the name arginine-vasotocin to the one in this group which possessed both vasopressor and oxytocic activity to a high degree. Katsoyannis and du Vigneaud (*61*) prepared arginine-vasotocin by utilizing a procedure similar to that employed for the synthesis of arginine-vasopressin. The azide of the *N*-terminal tripeptide, *S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-isoleucine, is coupled with the *C*-terminal hexapeptide, L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-arginyl glycinamide. The protecting groups are eliminated by treatment with sodium in liquid ammonia, and the product is oxidized by aeration. The substance is then purified by countercurrent distribution and zone electrophoresis. The pharmacological properties of the synthetic substance are similar to those of the natural hormone (*37*).

D. EVOLUTION IN THE DOMAIN OF THE NEUROHYPOPHYSEAL HORMONES

The data accumulated from the chemical as well as the pharmacological point of view are now sufficient to warrant the conclusion that the neurohypophyseal hormones are present in most vertebrates. Appreciable amounts of oxytocic and of vasopressin activities are found in the hypophysis of animals belonging to the different classes (cf. Table II). The cartilaginous fish exhibit the smallest quantities. The analysis of the active principles of certain species of mammals, birds, batrachians, and fish showed that hormones of different origin have extremely similar structures. However, this family of substances, which is so homogeneous on the chemical level, does not appear to play a single physiological role in the vertebrate kingdom. On the contrary, the organs on which they act vary from class to class, and among the fish their function is still unknown (*62, 63*). It is, therefore, difficult to speak of an evolution comprising structural modifications of the hormonal agent adapted to modifications of a specific receptor. At most it may be stated that in almost all classes there are two principal active principles and that, moreover, a large number of known biological activities may be related either to reproductive function or hydromineral regulation. At the present stage of our knowledge, then, it seems preferable to deal separately with the molecular transformations of the hormones, which represent variations in biosynthesis and which are the precise result of genetic mutations, and to consider later the possible evolution of the physiological functions involving several organs.

Very little is known about the biosynthesis of the neurohypophyseal hormones. They are manufactured in the hypothalamus and then trans-

TABLE II
BIOLOGICAL ACTIVITIES OF POWDERS OF THE POSTERIOR PITUITARY OF SOME VERTEBRATES

Animal	Oxytocic activity (U.S.P. units/mg.)	Vasopressor activity (U.S.P. units/mg.)
Mammals		
Beef	1.0	1.1
Hog	1.4	1.4
Horse	0.9	0.8
Sheep	0.8	0.8
Whale	0.5	2.5
Rat	3.2	3.2
Birds		
Fowl	0.4	0.4
Batrachians		
Frog	1.2	1.0
Bony fish[a]		
Hake	0.5	0.3
Pout	0.5	0.5
Pollack	0.5	0.5
Cartilaginous fish[a]		
Ray	0.005	Depressive
Dogfish	0.010	0.007

[a] Powder of the entire hypophysis.

ported to the neurohypophysis (*1, 64*), where they accumulate. With regard to precursors and synthetic reactions, we are reduced to the same hypotheses as those adopted for the biosynthesis of peptides and proteins. It has long been noted that the content of hormones in the neurohypophyseal tissue varies in parallel with that of a substance which stains with chrome hematoxylin and which is often called the Gomori substance (*1, 64*). It is also known that often the hormones are associated with a protein, *neurophysin* (*65*). Is the latter the Gomori substance and does it have a role during the formation of the active principles? It is not possible to answer these questions.

In considering the structures of the four hormones isolated up to the present, one is struck by the fact that, with a fundamental motif of nine residues, variations involve only one, or at most two, residues and that these variations always concern two privileged positions in the chain (position No. 3 and position No. 8) (cf. Fig. 2). Modifications at privileged points have been observed in other families of hormonal peptides, e.g., the insulins.[*] This "mutability" of certain portions of the peptide

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[*] See Section IV,C (Table IV).

1-2-Ileu-4-5-6-7-Leu-9	Oxytocin
1-2-Ileu-4-5-6-7-Arg-9	Arginine-vasotocin
1-2-Phe-4-5-6-7-Arg-9	Arginine-vasopressin
1-2-Phe-4-5-6-7-Lys-9	Lysine-vasopressin

FIG. 2. Variations in structures of the neurohypophyseal hormones in positions 3 and 8 of the peptide chain.

chain cannot fail to recall the mutability of certain genes, and the question may be raised whether this does not reflect a more general mechanism of transformation of protein molecules, a mechanism which had a role during evolution. It must, however, also be noted that, in most of the species studied, two "sister hormones" have been demonstrated and in some instances isolated (mammals, fowl). It is conceivable that a distant ancestor of the vertebrates may have possessed a single peptide and that a duplication of the gene governing its synthesis may have given rise to twin peptides. These two peptides may have been at the origin of two lines of molecules formed by independent mutations, whose terminal representatives in the mammals are oxytocin and arginine-vasopressin. Since the mutations occur at random, only those derivatives are retained which are useful to the organism, but it is not obligatory that all the hormones formed in this way must have had as target the same cell or the same organ. Nevertheless, before formulating an interpretation of the whole, it is necessary to isolate and identify the second active principle demonstrated in the batrachians and fish in order to have a sufficiently complete scheme of the neurohypophyseal hormones of the vertebrates.

The hydromineral regulation of the organism poses quite different problems in salt-water fish, the freshwater fish, amphibians, and land vertebrates. The kidney plays a preponderant role in mammals, but the gills in fish, the skin and the bladder in amphibians, and the cloaca in birds also participate in the exchanges of water and salts. The regulation brings into play specialized hormones, and it may be asked whether modification of the receptor automatically entails modification of the stimulating agent. Arginine-vasotocin has been isolated in marine bony fish, the frog, and the fowl. Its role in the maintenance of osmotic pressure in the first of these groups is obscure. The injection of pituitary extract of fish does not appear to release an antidiuresis, although this reaction can be inhibited by a "stress" which in marine teleosts leads to a "laboratory diuresis" (*62*). The involvement of hypophyseal hormones in the osmoregulation in a bony fish is suggested by the observation of Pickford (*66*): hypophysectomized *Fundulus heteroclitus*, in contrast to normal controls, cannot survive in fresh water whereas it can continue to live in salt water. Moreover, as pointed out by Baldwin (*67*), freshwater teleosts bring into action mechanisms of regulation which are the inverse of those of salt-water teleosts, and it may be inquired whether adaptation is effected not only by modification of organs, but also by modification of hormones. In the frog, arginine-vasotocin acts on the skin, the renal tubule, and the bladder. Its sodium-transferring action (natriferic action) on the skin (*68*) could be interpreted as protection against the action of a hypotonic medium, the sodium equilibrating the passive entry of water into the animal under aquatic conditions. But it may also be noted that arginine-vasotocin acts on the renal tube and the bladder to favor the recovery of water by the organism (*12, 69*) and that the hormone participates rather in the struggle against desiccation when the animal is away from the water. In birds, arginine-vasotocin has clearly an antidiuretic action which involves the renal tube. In mammals, this hormone is absent, but a new one, generally arginine-vasopressin, is found which acts strongly on the renal tube at the level of the loop of Henle, enabling these animals to excrete hypertonic urine. It is of interest to note that arginine-vasopressin has a weaker antidiuretic action than arginine-vasotocin in the fowl (*13*) and in the frog (*69*), whereas the opposite is true in the dog (Table I). There is then in vertebrates a certain adaptation of the hormone to function, but it is difficult to say whether the evolution of the latter is attributable more to improvement in the receptor or to modification of the stimulator.

III. Group of the Melanocorticotropic Hormones

It has long been known that the hypophysis contains substances which can stimulate the adrenal cortex (adrenocorticotropic hormones, ACTH or corticotropins) and substances which cause the dispersion of the melanin in the pigmented cells of lower vertebrates (melanocyte-stimulating hormones or MSH's). Although the former are localized in the anterior lobe and the latter in the intermediate lobe, a lengthy controversy has arisen as to whether one single hormone is responsible for the two types of activity. The isolation of the active principles, in fact, demonstrated a close chemical relationship between the MSH's and the corticotropins; this relationship is expressed on the biological level by relatively similar properties: hog corticotropin acts on the adrenal cortex of the rat and on the melanocytes of the frog. The role of the MSH's in mammals remains unknown: are they actually functional hormones or rather fossil molecules which in primitive vertebrates had played a role similar to that now fulfilled by corticotropin in the higher vertebrates? Until the present, only substances derived from mammals have been purified. In most of the species studied, three factors, called α-MSH, β-MSH, and corticotropin, have been isolated. But while the

MSH's appear to be without effect on the adrenal cortex of the rat, the three products stimulate the melanocytes of the frog, and it would be of interest to establish whether this action is not accompanied by a profound physiological effect. During embryological development, the anterior and intermediate lobes of the hypophysis have the same origin and the specialization of the synthetic processes perhaps was not always so clear cut.

Be that as it may, the structural analogy on the one hand and the similarity of certain biological effects on the other appear to justify the simultaneous consideration of the α-MSH's, β-MSH's and corticotropins; interpretation of the chemical resemblance between the three families on the phylogenetic or physiological level remains for the future.

A. BIOLOGICAL PROPERTIES

The substances which are commonly used for the biological assays are derived from mammalian hypophyses. It is important to note that structural variations have been established between β-MSH or corticotropins obtained from various species of this class of vertebrates and that it is not unreasonable to assume that differences may exist between the hormones currently used and those present in the lower vertebrates.

1. *Action in Mammals*

a. Action on Adrenal Ascorbic Acid. Corticotropin causes a decrease in adrenal ascorbic acid. Sayers *et al.* (*70*) have made use of this property to develop a practical assay for this hormone. The left adrenal gland of a hypophysectomized rat is removed immediately prior to the injection of the solution to be assayed, and the right gland is removed 1 hour later. The difference in ascorbic acid content between the two glands permits an assessment of the quantity of active principle injected. The relation existing between the decrease in ascorbic acid and the secretion of corticosteroids which occurs simultaneously is not known. It is interesting to note that mammalian corticotropin does not exert any action on the ascorbic acid content of the adrenal glands of birds (*71–73*) and batrachians (*74*) although it causes the secretion of corticosteroids in the fowl (*73*).

b. Corticosecretory Action. Corticotropin stimulates the secretion of corticosteroids by the adrenal tissue, and it is possible to assay the hormone by determining the quantity of steroids liberated. The assay can be carried out *in vitro* by allowing the hormone to act on the isolated gland (*75*) or *in vivo* by measuring the increase in corticosteroids in the plasma following the injection of corticotropin (*76*).

2. *Action in Birds*

Corticosecretory Action. In the fowl, the injection of mammalian corticotropin causes an increase in corticosteroids in the adrenal vein blood (*77*). The secreted products are identical with those in mammals.

3. *Action in Lower Vertebrates*

a. Corticosecretory Action. The adrenal glands of reptiles, batrachians, and fish secrete corticosteroids, and the stimulatory action of corticotropin has been observed in a snake (*77*).

b. Melanotropic Activity. Mammalian MSH's and corticotropins act on certain pigmented cells, the melanocytes, which are abundant primarily in the lower vertebrates. One observes a dispersion of melanin or a multiplication of the cells, effects which are expressed by a darker coloration of the animal. Biological assays *in vivo* and *in vitro* have been developed in the batrachians. Landgrebe and Waring (*78*) used *Xenopus:* the solution to be assayed is injected in the dorsal lymph sac and the degree of "dilatation" of the melanocytes of the interdigital membrane is determined microscopically. Shizume *et al.* (*79*) measured the change in color of isolated frog skin: the skin, stretched on a ring, is placed in a physiological solution to which the hormone is added. The change in color is measured by reflectometry. In certain fish, particularly *Fundulus,* hog β-MSH and sheep corticotropin cause an increase in the number of pigmented cells (*80*).

B. PURIFICATION

Until the present, only mammalian hormones have been isolated. In most of the species investigated, three active principles have been found, each factor varying slightly from species to species. It is, then, possible to distinguish three families of hormones: the α-MSH's, the β-MSH's, and the corticotropins. Although purification has been accomplished in the order corticotropins, β-MSH, α-MSH, it is preferable to follow the inverse order in this discussion since it corresponds to an increasing complexity of the molecules.

1. *Purification of the α-Melanocyte-Stimulating Hormones*

The first investigations were carried out on powders of hog posterior pituitary because they are two to five times as rich in melanotropic activity as those of beef. Lee and Lerner (*81*) showed that the gland contains two factors: a basic peptide, α-MSH, responsible for three-quarters of the activity of the tissue, and β-MSH, responsible for the rest. The two substances, having different solubilities and charge, can be separated by countercurrent distribution (*81*) and electrophoresis (*82*). The purification procedure for α-MSH comprises four stages (*81*): extraction of the acetone powder by glacial acetic acid at 50° and fractional precipitation with acetone and petroleum ether, adsorption onto hydroxycellulose according to the method introduced by Astwood *et al.* (*83*) for the purification of corticotropin, countercurrent distribution in a system of secondary butanol-0.5% trichloroacetic acid, and finally paper electrophoresis with pyridine acetate as the buffer. The yield is of the order of 5–15%. The pure product has a melanotropic activity of the order of 3000 I.U. per milligram. The isoelectric point is 10.5–11. An analogous procedure was used for the purification of the α-MSH of beef (*84*), horse (*85*), and monkey (*86*); in the latter instance, chromatography on carboxymethylcellulose replaced countercurrent distribution. Chromatographic methods that make it possible to deal with appreciable quantities of material have been proposed (*87, 88*). αMSH has been demonstrated in man, but its very low concentration has forestalled isolation (*86*).

2. *Purification of the β-Melanocyte-Stimulating Hormones*

The purification of β-MSH has been carried out in three laboratories using very similar methods. Landgrebe and Mitchell (*89*) and Porath *et al.* (*90*) made the extraction of the powder of the posterior pituitary with glacial acetic acid and the fractional precipitation by means of acetone and ether, then adsorption onto hydroxycellulose, and finally two exposures to zone electrophoresis on a cellulose column with pyridine acetate buffer. Benfey and Purvis (*91*) used a procedure in which the first two operations are identical with those above, but in which countercurrent distribution replaces zone electrophoresis. Geschwind and Li (*92*) utilized zone electrophoresis on a starch trough and countercurrent distribution in a system of secondary butanol-0.5% trichloroacetic acid. Hog β-MSH has an isoelectric point of 5.2–5.8. The pure product possesses melanotropic activity of the order of 2000 I.U./mg.

β-MSH has also been isolated from the glands of beef (*93*), monkey (*86*), and man (*94*). The procedure used for the purification of the hormone of beef is essentially the same as that utilized by Geschwind and Li for the hormone of hog. The isoelectric point of beef β-MSH is 7 instead of 5.8, the difference in charge arising from the replacement of a glutamic acid residue by a serine residue. Monkey β-MSH was purified by Lee *et al.* (*86*) by chromatography on carboxymethylcellulose. It differs from beef β-MSH by the replacement of a lysine residue by an arginine residue. Finally, human β-MSH, isolated by Dixon (*94*) by chromatography on Zeo-Karb 225 in the presence of urea and by paper electrophoresis, has been shown to be quite different from the other three. There are four additional amino acid residues; moreover, a lysine residue is replaced by an arginine residue as in monkey β-MSH.

3. *Purification of the Corticotropins*

The purification of hog corticotropin was undertaken first because the anterior pituitary powder of this species is 4–5 times as rich in corticotropic activity as that of beef. It was believed for several years that the hormone was a protein with a molecular weight of 20,000, but chromatographic methods showed that this was an aggregate and that the active principle in reality was a peptide with a molecular weight of about 4500. All the investigators who succeeded in obtaining the hormone employed the specific adsorption of the active principle on hydroxycellulose introduced by Astwood and his collaborators (*83*): the anterior pituitary powder is extracted by glacial acetic acid at 70° and the hormone is first purified by fractional precipitation with acetone and ether; the active material is dissolved in 0.1 *N* acetic acid and the solution is passed through a hydroxycellulose column which retains all the corticotropic activity and only about 10% of other substances. The hormone is then eluted with 0.1 *N* HCl. Shepherd *et al.* (*95*) used this procedure as the first operation, then carried out a countercurrent distribution of the active fraction in the system 6% acetic acid-3.5% NaCl-normal butanol. Eight active constituents were demonstrated, but only the principal constituent was purified by a second distribution and characterized completely. The other active products may be derived from the principal constituent by modifications due to the extraction. White (*96*) isolated hog corticotropin after adsorption onto hydroxycellulose, chromatography on Amberlite XE-97, and countercurrent distribution in the system secondary butanol-0.2% trichloroacetic acid. Dixon and Stack-Dunne (*97*) also used chromatography on Amberlite IRC-50 as the final purification step; they placed in evidence several active components, A_1, A_2, A_3, with A_2 possibly derived from A_1 by deamidation. Until now, only a single corticotropin has been characterized in the hog, and it is not certain that other active products exist normally in the gland. Hog corticotropin (called A-, A_1-, or β-corticotropin, according to the authors) possessed in an intrinsic fashion both corticotropic activity in the rat and melanotropic activity in the frog.

The corticotropins of sheep (*98*), beef (*99*), and man (*100*) have also been obtained. Li *et al.* (*98, 99*) purified the sheep and beef hormones by electrophoresis, chromatography, and countercurrent distribution. Human corticotropin was isolated by Lee *et al.* (*100*) using primarily two sequences of chromatography on diethylaminocellulose and carboxymethylcellulose. These three corticotropins have the same amino acid composition but differ from that of hog in that they have an additional serine residue and are missing a leucine residue. Besides, there are slight structural variations from one species to the next.

C. Chemistry

1. *Family of the α-Melanocyte-Stimulating Hormones*

This family is composed of peptides with 13 residues having an acetylated *N*-terminal end and an amide *C*-terminal end. The α-MSH's of hog, beef, horse, and monkey have identical structures so that a single molecular type has been demonstrated so far.

a. Structure. The structure of hog α-MSH was established by the investigations of Harris (*101*). Amino acid analysis indicates the presence of 13 residues. The classic techniques used for the identification of the *N*-terminal end (phenylisothiocyanate and dinitrofluorobenzene) and of the *C*-terminal end (carboxypeptidase) show that the ends are not free. Trypsin and chymotrypsin hydrolysis yielded, respectively, two and five fragments whose structure was established either directly or by carrying out partial acid hydrolysis and identifying the products of degradation. The twelve peptide linkages were determined in this way. Moreover, a molecule of ammonia was liberated by hydrolysis of a fragment obtained with chymotrypsin which did not contain an acid residue, showing that the *C*-terminal end is amided. Finally, another peptide produced with chymotrypsin, which contained serine and tyrosine and whose *N*-terminal end was blocked, yielded acetyl hydrazide and serine hydrazide after hydrazinolysis, showing that it was acetylseryltyrosine, the *N*-terminal sequence of the molecule. This body of information led to the following formula for hog α-MSH:

$$\text{Acetyl}\cdot\overset{1}{\text{Ser}}\cdot\overset{2}{\text{Tyr}}\cdot\overset{3}{\text{Ser}}\cdot\overset{4}{\text{Met}}\cdot\overset{5}{\text{Glu}}\cdot\overset{6}{\text{His}}\cdot\overset{7}{\text{Phe}}\cdot\overset{8}{\text{Arg}}\cdot\overset{9}{\text{Try}}\cdot\overset{10}{\text{Gly}}\cdot\overset{11}{\text{Lys}}\cdot\overset{12}{\text{Pro}}\cdot\overset{13}{\text{Val}}(\text{NH}_2)$$

The structures of the α-MSH's of beef (*84*), horse (*85*), and monkey (*86*) have been determined and have turned out to be identical with that of hog α-MSH.

b. Synthesis. The synthesis of α-MSH was accomplished by Boissonnas and his collaborators (*102–104*) and Hofmann and his team (*105*). This synthesis is particularly interesting because on the one hand it confirms the structure of the hormone and on the other hand it constitutes the first steps in the synthesis of hog corticotropin, since the amino acid sequence of α-MSH represents the *N*-terminal sequence of corticotropin. Guttmann and Boissonnas (*102*) condensed *N*-acetyl-L-seryl-L-tyrosyl-L-seryl-L-methionyl-γ-benzyl-L-glutamate (*103*) with the L-histidyl-L-phenylalanyl-L-arginyl-L-trytophanyl-glycyl-ϵ-*N*-CBO-L-lysyl-L-prolyl-L-valylamide (*104*) and obtained the active principle upon elimination of the carbobenzoxyl and benzyl groups by hydrobromic acid in trifluoroacetic acid. The peptide was then purified by countercurrent distribution and paper electrophoresis at pH 5.8. The product obtained appears to be chromatographically and electrophoretically pure and possesses the physical and chemical properties of natural α-MSH. The synthetic hormone has an activity of 3300 I.U. per milligram, or the activity of the extracted hormone. For their part, Hofmann *et al.* (*105*) condensed the azide of the *N*-terminal pentapeptide bearing an acetylated amino group with the *C*-terminal octapeptide whose lysine residue had been either tosylated or formylated and in which the carboxyl group of valine was amided. A protected tridecapeptide was thus obtained which after purification by countercurrent distribution and electrophoresis on a cellulose block has a high degree of melanotropic activity. If the α-amino group of the tridecapeptide is blocked by a carbobenzoxyl rather than an acetyl group, the product also has biological activity (*105*). It is difficult to say whether these derivatives are active in themselves or whether the melanocytes liberate the blocked groups.

2. *Family of the β-Melanocyte-Stimulating Hormones*

This is a family of peptides composed in general of a chain of 18 amino acid residues whose amino and carboxyl ends are free. β-MSH has been isolated from five mammalian species. Slight structural differences have been found; the most important concerns human β-MSH which, as an exception, contains 22 residues because of the presence of an additional tetrapeptide on the *N*-terminal side.

a. Structure. Hog β-MSH was the first to be studied, and its structure was determined independently by Harris and Roos (*106*) and Geschwind *et al.* (*107*). The former authors, using the recurrent technique of Edman, succeeded in establishing the *N*-terminal decapeptide sequence; with the aid of carboxypeptidase, they identified aspartic acid in the *C*-terminal position. Hydrolysis with trypsin and chymotrypsin yielded peptides which were isolated by paper ionophoresis. The determination of the structure of the fragments was carried out by classic techniques, and the results made it possible to deduce the complete formula of the hormone. Geschwind *et al.* (*107*) also used the technique of Edman to determine the *N*-terminal sequence and carboxypeptidase, as well as hydrazinolysis to identify the *C*-terminal residue. Enzymatic hydrolysis yielded fragments whose characterization permitted the structure of β-MSH to be deduced (Fig. 3).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Hog		Asp·	Glu·	Gly·	Pro·	Tyr·	Lys·	Met·	Glu·	His·	Phe·	Arg·	Try·	Gly·	Ser·	Pro·	Pro·	Lys·	Asp
Beef		Asp·	*Ser* ·	Gly·	Pro·	Tyr·	Lys·	Met·	Glu·	His·	Phe·	Arg·	Try·	Gly·	Ser·	Pro·	Pro·	Lys·	Asp
Horse		Asp·	Glu·	Gly·	Pro·	Tyr·	Lys·	Met·	Glu·	His·	Phe·	Arg·	Try·	Gly·	Ser·	Pro·	*Arg*·	Lys·	Asp
Monkey		Asp·	Glu·	Gly·	Pro·	Tyr·	*Arg*·	Met·	Glu·	His·	Phe·	Arg·	Try·	Gly·	Ser·	Pro·	Pro·	Lys·	Asp
Man	*Ala·Glu·Lys·Lys*·	Asp·	Glu·	Gly·	Pro·	Tyr·	*Arg*·	Met·	Glu·	His·	Phe·	Arg·	Try·	Gly·	Ser·	Pro·	Pro·	Lys·	Asp

Fig. 3. Family of the β-MSH's.

With the aid of similar techniques, the structure of the β-MSH of beef (*108*), horse (*109*), monkey (*86*), and man (*110*) has been determined. Figure 3 shows the formulas and demonstrates the differences among the species. These differences consist in general in the substitution of a single residue: in beef, a serine residue in place of a glutamic acid residue in position 2; in the horse, an arginine residue in place of a proline residue in position 16; in the monkey, an arginine residue in place of a lysine residue in position 6. In man, on the other hand, there is an additional tetrapeptide

Ala·Glu·Lys·Lys

on the *N*-terminal side and, moreover, a lysine residue is substituted for an arginine residue.

It is of interest to note that the substitutions affect the melanotropic activity since the beef hormone is about ten times less active on the frog melanocytes than the hog hormone (*111*). Although the real function of the substances in mammals remains unknown, these substitutions may be interpreted as an adaptation for function. With regard to the human hormone, which contains an additional tetrapeptide, it is possible that the latter may be a residual fragment of an as yet unknown precursor. In fact, the removal of this tetrapeptide transforms human β-MSH into monkey β-MSH. It may be postulated that the cleavage which usually occurs in front of the aspartic acid residue may not have taken place in the formation of the human hormone, perhaps because the accumulation of charges around the bond to be broken

$$\text{Lys·Lys}\overset{\downarrow}{\cdot}\text{Asp·Glu}$$

has inhibited the action of the specific enzyme. In this case, a variation in the mechanism of liberation rather than in the mechanism of biosynthesis would be involved.

b. Synthesis. The synthesis of beef β-MSH was undertaken by Schwyzer and his collaborators (*112, 113*). The Ciba group first prepared the *N*-terminal heptapeptide, the central hexapeptide, and the *C*-terminal pentapeptide. The two latter fragments were condensed by the dicyclohexyl carbodiimide method; then, after liberation of the terminal α-amino group by catalytic reduction, they condensed the undecapeptide with the *N*-terminal heptapeptide. The octadecapeptide obtained has its amino and carboxyl groups blocked by protecting radicals, but it has, nonetheless, melanotropic activity of the order of 1% of the natural product. It is of interest to observe that the intermediate undecapeptide also has melanotropic activity, although it is lower than that of the final product.

Further, the liberation of the *N*-terminal amino acid of the intermediate undecapeptide by catalytic reduction does not appear to increase the biological activity to any significant degree.

3. *Family of the Corticotropins*

The corticotropins constitute a family of peptides consisting of a peptide chain of 39 residues. The corticotropins of hog, sheep, beef, and man have been isolated, and slight variations have been shown to exist from species to species.

a. Structure. Hog corticotropin was studied in the laboratories of the American Cyanamid Company (β-corticotropin) on the one hand and in those of Armour (corticotropin A) on the other, while the corticotropins of sheep and beef were the objects of investigations of Li and his collaborators at Berkeley. The structure of this particularly long polypeptide was approached by the usual methods: Shepherd *et al.* (*114*) and White and Landmann (*115*) carried out hydrolysis of hog corticotropin with trypsin, chymotrypsin, and pepsin, and the fragments obtained were purified by countercurrent distribution. The characterization of the peptides resulting from the degradation enabled the two groups of investigators to propose a complete formula differing merely in the position of four residues. Further studies (*116*) make it possible to settle the latter question so that the complete structure of hog corticotropin is now completely established (Fig. 4).

Li and his team, using the same techniques, determined the structure of the corticotropins of sheep (*117*) and of beef (*99*). The amino acid composition of the two hormones is identical, but the sequence of the residues varies between position 25 and 32 (Fig. 4). Finally, Lee *et al.* (*100*) isolated human corticotropin and studied its structure. The amino acid composition is identical with that of sheep or beef corticotropin. The first results indicated that its structure must be very similar to that of these hormones since the peptides obtained by trypsin hydrolysis resemble those obtained with the other corticotropins under analogous conditions. It is worth observing that the changes in structure occur in a portion of the molecule (residue 25 to residue 32) which is not indispensable for corticotropic activity. Figure 4 presents the formulas of hog, sheep, and beef corticotropins.

b. Synthesis. The observation that the corticotropic activity of corticotropin is not destroyed by pepsin (*118*) and that a fragment of 24 residues resulting from hydrolysis with pepsin and then acid has an activity equal to that of the intact hormone (*114*) encouraged the chemists to synthesize this fragment. The synthesis assumes added interest when it is pointed out that the 13-residue sequence of hog α-MSH repre-

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39

Hog Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val·Gly·Lys·Lys·Arg·Arg·Pro·Val·Lys·Val·Tyr·Pro·Asp·Gly·Ala·Glu·Asp·Glu·Leu·Ala·Glu·Ala·Phe·Pro·Leu·Glu·Phe

Sheep ←——— *ibid.* ———→Ala·Gly·Glu·Asp·Asp·Glu·Ala·Ser·←——— *ibid.* ———→

Beef ←——— *ibid.* ———→Asp·Gly·Glu·Ala·Glu·Asp·Ser·Ala·←——— *ibid.* ———→

FIG. 4. Family of the corticotropins.

sents the *N*-terminal sequence of the corticotropin of the same species. Boissonnas *et al.* (*119, 120*) synthesized an icosapeptide representing the sequence of the first 20 residues of corticotropin, but the corticotropic activity *in vitro* of this peptide was slight (2–3 I.U. per milligram). However, several years later, Li *et al.* (*121*) obtained a nonadecapeptide having corticotropic activity *in vivo* and *in vitro* of about 30 I.U. per milligram, and it became evident that the low activity observed by the Swiss authors must have been due to racemization. Hofmann *et al.* (*122*) finally synthesized a tricosapeptide representing the sequence of this first 23 residues of hog corticotropin and having an activity of 103 I.U. per milligram *in vivo* (depletion in adrenal ascorbic acid and rise in plasma corticosterone). These authors condensed the *N*-terminal decapeptide with the *C*-terminal tridecapeptide with the dicyclohexyl carbodiimide method. The protecting groups were eliminated by treating the resultant tricosapeptide with 0.5 *N* HCl at 100°, and the hormone was purified by chromatography on carboxymethylcellulose. The amino acid analysis corresponds to the expected values, and the biological activity of the product resembles that of natural corticotropin. The results of the synthesis, therefore, confirm the conclusions of Shepherd *et al.* (*114*) which indicated that the *C*-terminal sequence of corticotropin is not necessary for biological activity. The synthesis of a tetracosapeptide representing the *N*-terminal sequence of corticotropin has also been accomplished by Kappeler and Schwyzer (*122a*).

D. Relationships between Biological Activities and Structure

The three families of melanocorticotropic hormones present a structural analogy with regard to a sequence of 11 residues. The relation between the α-MSH's and the corticotropins is particularly close: the same α-MSH has been found in all the mammalian species studied and, moreover, if it is agreed that the *N*-terminal sequence of the first 23 residues of corticotropin possesses the biological activity of the entire molecule, the same "fundamental corticotropin" is found in hog, sheep, beef, and probably man. In the family of the β-MSH's, a slight variation from species to species is seen; generally, this involves a change in residue 18, except in human β-MSH, where an additional tetrapeptide is found (Fig. 3). The significance of these variations for the biological activity is difficult to assess since there are few quantitative data on the specific activity of the different β-MSH's; the only fact known is that beef β-MSH is about 10 times less active than hog β-MSH on the frog melanocytes, the lowering of activity corresponding to the replacement of a glutamic acid residue by a serine residue. It seems more instructive to consider the three hormones found in the hog and to examine the relations existing between these three structures on the one hand and the two biological activities, melanotropic activity and corticotropic activity, on the other.

In the first place, it should be observed that the three hormones have melanotropic activity whereas only corticotropin has corticotropic activity; this shows that the structure necessary for the first activity is less rigorously defined than that required for the second. The structural variations consist in lengthening of the peptide chain on the one hand, in possible blocking of the terminal amino and carboxyl groups on the other.

The investigations devoted to the synthesis of the different hormones have demonstrated the influence of a lengthening of the peptide chain on melanotropic activity. The pentapeptide

His·Phe·Arg·Try·Gly

which is found in all the hormones of the group, already has appreciable activity, and this activity increases proportionately as the peptide chain becomes longer (*121, 123, 124*) (cf. Table III). However, the tridecapeptide corresponding to α-MSH has only slight activity, and it is necessary that the *N*-terminal amino group be acetylated before the maximum activity of 3300 units is attained (*102, 125*). The lengthening of the chain on the *C*-terminal side up to 19 residues (corresponding to the sequence of the first 19 amino acids of corticotropin) causes only a slight increase in the melanotropic activity, and this melanotropic activity is almost equal to that of intact corticotropin (*121*). In contrast, lengthening of the chain on the *N*-terminal side, as in the case of β-MSH, yields a highly active molecule. It appears that the presence of a free amino group in proximity to the "active" sequence is detrimental and that melanotropic activity increases considerably when this group is blocked either by an acetyl radical or by an additional peptide. In support of this hypothesis, Waller and Dixon (*126*) observed that if the amino group of corticotropin is acetylated, the melanotropic activity of the peptide is increased five- to tenfold.

As far as corticotropic activity is concerned, the presence of a specific sequence of at least 19 residues appears to be necessary for the molecule to exhibit this property, since this activity goes from 29 to 103 I.U. per milligram when the number of residues increases from 19 to 23. According to Hofmann *et al.* (*122*), the tricosapeptide representing the sequence of the first 23 residues of corticotropin possesses the corticotropic activity of the intact corticotropin (39 residues). Consequently, a slight lengthening of the chain causes the activity to go from minimum to maximum, in contrast to what has been observed for melanotropic activ-

TABLE III

Relation between Melanotropic Activity and Chemical Structure (*102*, *123*, *124*)

Peptide	Structure	Activity[a] (I.U./mg.)
Pentapeptide	His·Phe·Arg·Try·Gly	0.003
Hexapeptide	Glu·His·Phe·Arg·Try·Gly	0.020
Octapeptide	NH$_2$ $\vert$ Ser·Met·Glu·His·Phe·Arg·Try·Gly	0.070
Hexapeptide	NH$_2$ $\vert$ Ser·Met·Glu·His·Phe·Arg	Inactive
Trideeapeptide	Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val(NH$_2$)	Weak
Acetyl trideeapeptide (hog α-MSH)	CH$_3$CO·Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val(NH$_2$)	3300
Nonadecapeptide (*N*-terminal sequence of hog corticotropin)	Ser·Tyr·Ser·Met·Glu·His·Phe·Arg·Try·Gly·Lys·Pro·Val·Gly·Lys·Lys·Arg·Arg·Pro	10
Octadecapeptide (hog β-MSH)	Asp·Glu·Gly·Pro·Tyr·*Lys*·Met·Glu·His·Phe·Arg·Try·Gly·*Ser*·Pro·Pro·Lys·Asp	2000

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[a] The activities were calculated in International Units on the basis of the equivalence 1 I.U. = 10^4 Shizume units [cf. I. I. Geschwind and C. H. Li, *J. Am. Chem. Soc.* **79,** 615 (1957)].

ity. It is a strange finding, however, that whereas the *C*-terminal portion of the native molecule is not necessary for activity and can be eliminated without apparent detriment (*114, 122*), the removal of the *N*-terminal residue by leucine aminopeptidase inactivates the hormone (*127*).

If consideration is now given to how the melanotropic and corticotropic activities evolve as the hormones are modified, a certain antagonism is noted between the structures required for these two properties: the modifications which augment one diminish the other. α-MSH has strong melanotropic activity and practically no corticotropic activity. The elimination of the acetyl radical which blocks the N-terminal amino group greatly reduces the melanotropic activity on the one hand, but causes corticotropic activity, on the other, to appear (*125*). Conversely, if the *N*-terminal amino group of corticotropin is acetylated, it is noted that corticotropic activity becomes ten times lower while melanotropic activity shows a tenfold rise (*126*). The deamination of the *N*-terminal group by periodate destroys corticotropic activity but does not modify melanotropic activity (*128*). Thus it seems that the mechanisms of action at the level of the receptor cells are different.

The presence of α-MSH and β-MSH in mammals until now has not been related to any precise function. In contrast, the action of corticotropin on the adrenal gland is of fundamental importance since the corticosteroids secreted by the latter are indispensable for the survival of the organism. The question may be raised whether α-MSH and β-MSH, related chemically to corticotropin but less complex, may not be fossil molecules identical or very similar to those which in primitive vertebrates played the role of the actual corticotropin. In this regard, it would be interesting to know whether α-MSH and β-MSH, which do not have corticotropic activity in mammals, exhibit such activity in, for example, batrachians or reptiles. The elegant techniques developed by Jones *et al.* (*77*), which made it possible to assess the action of mammalian corticotropin in the fowl and in a snake by identification and assay of the corticosteroids of adrenal venous blood, make these experiments possible. On the chemical level, the isolation and characterization of the melanocorticotropic hormones of the lower vertebrates may throw new light on the biological significance as well as the phylogenesis of the active principles of the group.

IV. Group of the Insulins

Ever since its discovery the importance of insulin on the clinical level has led investigators to purify this hormone and to study its constitution. The remarkable studies of Sanger and his collaborators led not only to the discovery of a group of techniques now in current use in the study of proteins, but also to the establishment of the complete structure of the insulins of several species of mammals. Beef insulin, in particular, has become a reference polypeptide for chemists which serves to test out new analytical techniques. In the field of comparative biochemistry, the substitutions of residues in *privileged positions* of the peptide chain have been demonstrated among insulins of different origin, and this demonstration may be compared to observations made in the group of neurohypophyseal hormones and that of melanocorticotropic hormones.

A. Biological Properties

As yet the insulins obtained in a high state of purity have been mammalian insulins. However, the physiology of these substances has been studied in the various classes of vertebrates. Since the removal of the pancreas causes hyperglycemia in almost all animals, one may expect to find insulin in all vertebrates (*129*).

1. *Action in Mammals*

The different effects of insulin have led to the development of several biological assays which were recently reviewed critically by Stewart (*130*).

a. Hypoglycemic Action. Insulin injected into an animal causes a decrease in blood glucose. An assay for insulin based on this action has been developed in the rabbit. The percentage of the decrease is proportional to the logarithm of the dose. The hormone is injected subcutaneously and the samples of blood are taken one-and-a-half hours later from the external ear vein (*130, 131*).

b. Convulsive Action. The injection of insulin into mice results in convulsions due to hypoglycemia if the animals are maintained at elevated temperatures. The percentage of animals exhibiting convulsions rises linearly as a function of the logarithm of the dose, and the hormone may be assayed in this way (*130, 131*).

c. Action on the Formation of Muscle Glycogen. Insulin increases the glycogen content of isolated rat diaphragm placed in a solution containing glucose. There is a relationship between the quantity of glucose fixed by the tissue and the quantity of insulin added to the medium; this relation makes the assay of the hormone feasible. The method is very sensitive and can be used for the assay of insulin in blood (*131, 132*).

d. Action on Lipid Formation. Insulin favors the fixation of glucose and its oxidation with the formation of CO_2 and incorporation of certain carbon atoms into the lipids of rat epididymus maintained in a suitable buffer. If glucose-1-C^{14} is used, it is possible to collect the CO_2 liberated as $BaC^{14}O_3$ and to measure the radioactivity. There is a linear relation between the square root of the number of counts per minute and the logarithm of the dose of insulin added to the medium (*131, 133*).

c. Action on Protein Formation. The hormone accelerates the incorporation of radioactive amino acids into tissue proteins. An assay based on the incorporation of glycine-C^{14} into the proteins of rat diaphragm makes it possible to estimate very small quantities of insulin (*131, 134*).

2. *Action in Birds*

Insulin causes hypoglycemia in birds (*135*). However, in contrast to observations in the other vertebrates, the total removal of the pancreas causes an unexpected hypoglycemia. To explain this fact it has been pointed out that the elimination of the organ suppresses not only the source of insulin, but also that of glucagon, an antagonistic factor causing hyperglycemia, and that this hormone may have a predominant action in birds (*135*).

3. *Action in Lower Vertebrates*

Mammalian insulin causes hypoglycemia and convulsions in batrachians (*136*). In contrast, it has been reported not to bring about hypoglycemia in reptiles but since the commercial preparations of insulin are quite often contaminated with glucagon, the question may be raised whether this "insensitivity" to insulin is not merely an apparent one, a result of the particular hypersensitivity of these animals to glucagon (*136*).

B. PURIFICATION

Purification of the insulins still is based on the original procedure of Banting and Best. Romans *et al.* (*137*) carried out the extraction of fresh or frozen pancreas by means of 93% alcohol acidified with hydrochloric acid. The yield of the extraction can be improved by addition of sodium chloride (*138*). The solution, freed of insoluble material, is adjusted to pH 8; then, after elimination of the precipitate formed, it is brought to pH 2.0–2.5 and concentrated. The addition of sodium chloride to a concentration of 25% precipitates the active fraction. The precipitate is extracted with water at pH 2.0, and the active substance is again reprecipitated from the solution by sodium chloride at a concentration of 15%. The precipitate is then extracted under the previous conditions and upon adjustment of the solution to pH 5.0–5.4, insulin precipitates at its isoelectric point. The material is redissolved in dilute acetic acid; to the solution is then added about half its volume of acetone, the pH is adjusted to 5.9–6.0, and a solution of ammonium acetate at pH 5.9–6.0 is added. The addition of a solution of zinc acetate in suitable proportions causes the precipitation of the insulin, which is redissolved in dilute acetic acid and recrystallized at pH 5.9 in the presence of zinc acetate. The activity of the resultant crystals is about 24 I.U. per milligram.

A new method for the preparation of the insulins was proposed recently by Pettinga (*139*). Beef, hog, or dog pancreas is extracted with acid alcohol and the active fraction is precipitated with alcohol and ether at pH 3; the precipitate is redissolved in dilute sulfuric acid and the solution is seeded with fibrils prepared from insulin crystallized according to the method of Waugh *et al.* (*140*). The hormone precipitates in the form of fibrils and can then be crystallized.

The presence of several active constituents in crystalline beef insulin was shown by countercurrent distribution (*141*), electrophoresis (*142*), and ion-exchange chromatography (*143, 144*). However, Harfenist (*145*) was able to show that the two active constituents isolated by countercurrent differed only by an amide group and that two distinct molecular species were not actually involved.

The molecular weight of insulin has been the subject of very numerous studies. The value of 6000 found by certain physical methods (*146, 147*) is in agreement with the structural data provided by the chemical investigations.

C. CHEMISTRY

The complete structure of beef insulin has been established by Sanger and his collaborators in four stages: determination of the number of peptide chains making up the molecule, separation of the chains, determination of the amino acid sequence in the chains, and localization of the linkages between the chains.

1. *Determination of the Number of Chains*

To resolve this problem, Sanger (*148*) developed his well-known dinitrofluorobenzene method which makes it possible to estimate the number of *N*-terminal residues of proteins. Since the molecular weight accepted at the time was 12,000, it appeared that there were 2 glycine residues and 2 phenylalanine residues in the *N*-terminal positions and that, therefore, the molecule was composed of four chains. Actually, Harfenist and Craig (*146*) showed that the real molecular weight is near 6000, which reduces the number of chains to two.

2. *Separation of the Chains*

Having assumed that the chains would be united by the disulfide bridges of cystine residues, Sanger (*149*) broke these bridges by oxidation with performic acid. Each cystine residue yielded 2 cysteic acid residues. The separation of the chains was then carried out by fractional precipitation. Sanger found that one of them (chain A) did not contain basic amino acids and had about 20 residues whereas the other (chain B), more complex, was composed of about 30 residues.

3. *Determination of the Structure of the Chains*

In carrying out partial acid hydrolysis of the dinitrophenylated derivatives of chains A and B, Sanger (*150*) was able to isolate the *N*-terminal peptides because of the particular solubility in organic solvents conferred by the dinitrophenyl radical. The analysis of these peptides made it possible to establish the *N*-terminal sequence Gly·Ileu·Val·Glu·Glu of the A chain and the *N*-terminal sequence Phe·Val·Asp·Glu for the B chain. Sanger and Tuppy (*151, 152*) then succeeded in establishing the complete structure of the B chain by isolating the peptides of degradation obtained either by partial acid hydrolysis or by enzymatic hydrolysis. They showed that the concern about rearrangements, which until then had caused enzymes to be rejected as tools for degradation in structural studies, was not justified and that, moreover, these agents by reason of their specificity could furnish peptides different from those obtained by acid hydrolysis and thus could resolve certain problems of localization of residues which had resisted chemical techniques. In fact, the results obtained with the B chain of insulin opened the way to the use of enzymes as chemical agents for the hydrolysis of peptide chains and encouraged more audacious enterprises on chains of 120–150 residues. Sanger and Thompson (*153, 154*) established the sequence of the A chain with the same methods. The peptides obtained by enzymatic hydrolysis made it possible to determine the position of the amide groups of aspartic and glutamic acid residues found in the chains (*155*).

4. *Localization of the Disulfide Bridges*

Since the A chain contains 4 cysteic acid residues whereas the B chain contains 2, it is logical to envisage the existence, on the one hand, of 2 disulfide bridges connecting the 2 chains and, on the other, of a disulfide bridge uniting 2 residues of the A chain. To localize the interchain bridges, it is necessary to isolate peptides of nonoxidized insulin under conditions such that rearrangements of the disulfide bridges do not take place. Sanger and his collaborators (*156, 157*) found that these conditions are different depending on the pH, and they carried out acid and enzymatic hydrolyses with the avoidance of modifications. The peptides they obtained made it possible to localize the two interchain disulfide bridges. The complete structure of the hormone was thus determined (Fig. 5).

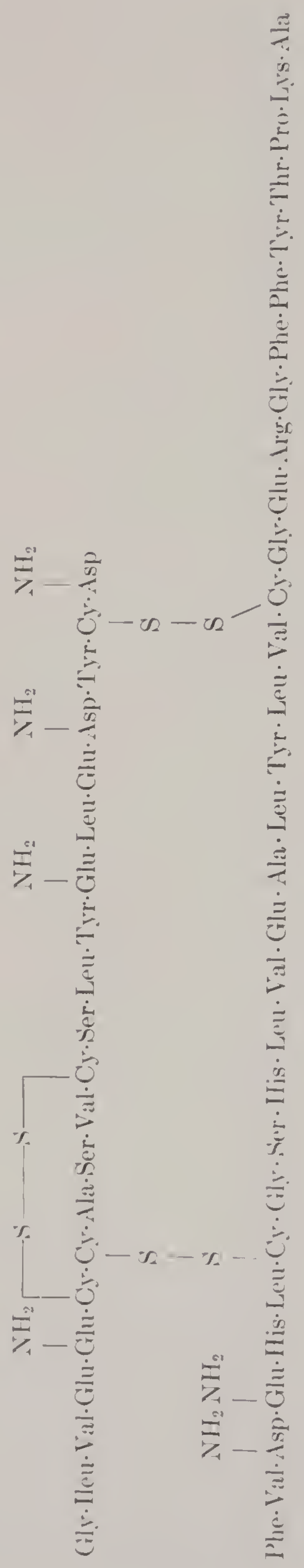
NH₂
S
S
NH₂
NH₂
NH₂
Gly·Ileu·Val·Glu·Glu·Cy·Cy·Ala·Ser·Val·Cy·Ser·Leu·Tyr·Glu·Leu·Glu·Asp·Tyr·Cy·Asp
S
S
S
S
NH₂ NH₂
Phe·Val·Asp·Glu·His·Leu·Cy·Gly·Ser·His·Leu·Val·Glu·Ala·Leu·Tyr·Leu·Val·Cy·Gly·Glu·Arg·Gly·Phe·Phe·Tyr·Thr·Pro·Lys·Ala

FIG. 5. Structure of beef insulin.

Although the insulins of different mammalian species have the same biological activity (*158*), the amino acid compositions of the insulins of beef, sheep, and hog exhibit some differences (*159*) and structural variations have actually been observed. At present, the hormones of eight species are known: beef (*157*), hog (*160*), sheep (*160*), horse (*161*), sperm whale (*161*), whale (*162*), man (*163*), and rabbit (*164*). All these insulins contain 51 residues and the variations involve only residues 8, 9, and 10 of the A chain and residue 30 of the B chain. Table IV shows the differences. Position 8 of the A chain is occupied by alanine or threonine, position 9 by serine or glycine, position 10 by valine or isoleucine, although in the whale a threonine residue is observed as an exception. Finally, in the B chain the alterations take place in the *C*-terminal residue, which may be alanine, threonine, or serine.

TABLE IV
DIFFERENCES IN STRUCTURE BETWEEN THE INSULINS OF SEVERAL MAMMALIAN SPECIES

	A chain, No. of residue			B chain, residue No.
Species	8	9	10	30
Beef	Ala	Ser	Val	Ala
Hog	Thr	Ser	Ileu	Ala
Sperm whale	Thr	Ser	Ileu	Ala
Man	Thr	Ser	Ileu	Thr
Rabbit	Thr	Ser	Ileu	Ser
Whale	Ala	Ser	Thr	Ala
Sheep	Ala	Gly	Val	Ala
Horse	Thr	Gly	Ileu	Ala

D. RELATIONS BETWEEN BIOLOGICAL ACTIVITY AND STRUCTURE

The insulin molecule appears to be strictly adapted to its biological function since until now most of the modifications which have been tried have inactivated the hormone. As the insulins of different mammalian species have essentially the same activity, it may be assumed that the residues in positions 8, 9, and 10 of the A chain and the residue 30 of the B chain, which vary from species to species, are not determined very rigorously. Further, the presence of residue 30 of the B chain, which occupies the *C*-terminal position, does not even seem to be indispensable since it can be eliminated with carboxypeptidase without disappearance of the activity (*165*). Trypsin, because of its specificity, cleaves in this molecule only two peptide bonds located in the B chain where a lysine and an arginine residue are found. One of these two bonds may be broken by carboxypeptidase without inactivation; the cleavage of the second, however, which causes the elimination of 8 residues from the *C*-terminal side of the B chain, brings about an 85% decrease in activity, but the residual insulin remains active (*166*). With regard to the A chain, it is not even possible to remove the C-terminal residue with carboxypeptidase without causing the loss of the activity (*165*).

On the *N*-terminal side, the experiments carried out by Smith *et al.* (*167*) with leucine aminopeptidase on zinc-free insulin suggest that the first 6 residues of the B chain are not necessary for biological activity. In contrast, the degradation of the A chain by the enzyme has an influence. The first studies on the localization of the "active center" of insulin with enzymatic methods thus seem to indicate that the integrity of the A chain is indispensable whereas the B chain may be shortened to some extent at its ends. However, it appears difficult to act in this fashion exclusively on one chain, leaving the other intact; the recent results on the reconstitution of insulin from its separated chains may open the way to modifications carried out with precision on one or the other part of the molecule. In the same way, chemical alterations (acetylation, esterification, iodination, etc.), if they are carried out on the entire insulin molecule, cannot provide direct information on the "active center" since they affect simultaneously several residues located in different sites of the molecule; however, it would be interesting to carry them out on the isolated chains and then to unite the modified fragments.

The problem of the separation of the chains and that of their reassociation in the proper position is particularly important since it bears not only on the study of the "active center" of the hormone, but also on its synthesis. When the rupture of the disulfide bridges that unite the two chains is carried out by oxidation, using for example performic acid, there occurs the formation of stable cysteic acid residues from cystine; this permits a convenient purification of the chains, but it becomes very difficult to envisage their reassociation. In contrast, the reduction of the bridges by thioglycolic acid, for example, which leads to the formation of cysteine residues, preserves the possibility of reconstitution but does not yield stable products; it is therefore necessary to transform the cysteine residues into particular derivatives to be able to purify the fragments. Nonetheless, if the stable derivatives are selected judiciously, it is possible to reconvert them to cysteine residues after separation and to attempt a reassociation of the chains. It is known from the work of du Vigneaud *et al.* (*168*) that the reduction of the disulfide bridges of insulin abolishes the hypoglycemic action of the hormone. However, reduced insulin retains the ability to raise the respiratory quotient of adipose tissue and to accelerate the conversion of glucose-C^{14} into fatty acids by surviving tissue (*169*). Langdon (*169*) reduced insulin completely with an excess of thioglycolate in the presence of 8 *M* urea, then converted the thiol groups to phenylmercuric mercaptides. He was then able to separate the two chains by zone electrophoresis. After purification and crystallization of the derivative of the B chain (phenylalanine chain), he regenerated the thiol groups by the action of cysteine in the presence of 8 *M* urea. The B chain thus obtained possessed activity on the respiratory quotient and on the incorporation of glucose-C^{14} into fatty acids. Moreover, this chain is an effective inhibitor of glutathione reductase, an activity not exhibited by intact insulin, and Langdon (*169*) formulated the hypothesis that the fragment may be the principle having an immediate metabolic effect, insulin being then a sort of "prohormone." The existence of an "insulin reductase" has been postulated (*170*) and its role in the activation of insulin has been envisaged (*169*), but this concept requires a solid experimental basis before being accepted.

Dixon and Wardlaw (*171*) separated the A and B chains of insulin by treating the molecule with sulfite. The two chains were obtained in the form of S-sulfonated derivatives which could be purified by ion-exchange chromatography in the presence of urea. The thiol groups of the chains were then regenerated by treating the S-sulfonated derivatives with cysteine or mercaptoethanol. The isolated chains did not exhibit biological activity when tested by either the method of convulsions in mice or that of stimulation of glycogen synthesis in diaphragm *in vitro*. However, if the reduction by mercaptoethanol is carried out on a mixture of S-sulfonated derivatives of the two chains, the appearance of biological activity is observed to the extent of 1–2%. Air oxidation after reduction does not appear to increase the yield of activity. Resynthesis of insulin has been demonstrated by comparing the active product and standard insulin by means of zone electrophoresis and an immunological method. Tsao Tien-Chin (*172*) also separated the A and B chains in the form of S-sulfonated derivatives and purified them by zone electrophoresis or chromatography. The purity of the resultant chains was checked chemically by amino acid composition and by determining the *N*- and *C*-terminal residues. The S-sulfonated groups were converted to thiol groups by thioglycolic acid or by treatment with sodium in liquid ammonia. Air oxidation of a mixture of A or B chains causes the appearance of the biological activity whereas the oxidation of the A chain or the B chain separately yields only inactive products. The purified active product has a specific activity of 18 I.U. per milligram, yields crystals identical with those of insulin, and after hydrolysis gives a "fingerprint" similar to that of insulin. It seems, then, that the dissociation of the chains can be carried out in a reversible manner and that the way is open for precise modifications in each chain as well as for the chemical synthesis of insulin. However, the union of the two portions of the molecule in the proper position does not appear to occur with a high yield and the problem of the directed union of the two chains still remains to be solved.

V. Conclusions

The field of protein hormones at first attracted the chemists because among these hormones there are relatively simple peptides; indeed, it was the studies carried out on small hormone molecules which opened the way for more ambitious enterprises which, at present, point toward the determination of the complete structure of an increasing number of proteins. But now that the analytical researches have been crowned with success, the simplicity of the hormone peptides can also be of value in the study of two great problems which preoccupy investigators currently: the mechanism of action of protein molecules on the one hand, their biosynthesis on the other.

In the first instance, it has been possible to modify the small molecules at precise sites of the structure by making use of enzymes or of specific reagents; to label certain amino acids and to follow the fate of the hormone; and, finally, to obtain analogs by synthesis and to determine, to a certain extent, the importance of certain residues for biological activity. However, hormonal action cannot actually be expressed in chemical terms, since the integrity of the target cell is required for the manifestation of activity. This near impossibility of developing subcellular or soluble systems renders the direct study of the mechanism of action of the hormones more difficult to approach than that, for example, of the mechanism of action of enzymes.

With respect to the biosynthesis of peptides and proteins, it is of interest to relate the observations made on the structure of currently known hormones to those obtained on other proteins. In all classes of the vertebrates investigated, there are at least two neurohypophyseal peptides built on the same structural motif, and in most mammals that have been examined three adenohypophyseal hormones have been demonstrated (αMSH, β-MSH, and corticotropin) which exhibit a striking chemical relationship. Moreover, it is known that trypsin and chymotrypsin have a structural similarity in the neighborhood of a serine residue important for activity, that the α- and β-chains of hemoglobin have a marked analogy, that there are two similar β-lactoglobulins in cow's milk, and, lastly, that the antibiotic peptides form a family of molecules having very similar structures, such as the tyrocidins A, B, and C which differ from each other by a single amino acid residue. The presence of two or three models of the same molecular motif in peptides or proteins having very different functions seems, therefore, to be a rather general phenomenon. It would be interesting to study in parallel the genetic systems controlling the syntheses of these related molecules. In particular, the question may be raised whether the primitive organ or cell may not manufacture only a single representative and whether later reduplication of the gene may not have involved that of the peptide or protein whose synthesis it controls. Investigations on hormones in the oldest vertebrates may indirectly yield information on the mechanism of formation of new molecules.

Acknowledgment

This review owes much to the suggestions and counsel of Professor Jean Roche, who has been kind enough to read the manuscript and to give it the benefit of his critique.

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CHAPTER 7

Comparative Biochemistry of Digestive Mechanisms

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I. Introduction 347
II. Digestion in General 348
  A. Secretion of Enzymes (Mammals) 348
  B. Absorption of Products of Digestion 349
  C. Role of Intestinal Movements 356
III. Digestion in Special Physiological and Systematic Groups of Vertebrates 356
  A. Role of the Mouth of Vertebrates in Digestion 357
  B. Stomach and Intestine of Monogastric Vertebrates 357
  C. Ruminants and Animals with Similar Modes of Digestion 361
  D. Birds 367
  E. Reptiles 372
  F. Amphibia 373
  G. Fishes 374
IV. Digestion in Invertebrates 377
  A. Phagocytosis and Intracellular Digestion 378
  B. Invertebrates with a Well-Developed Intestine 381
  C. Differences between Chemical Digestive Agents of Vertebrates and Invertebrates 382
  D. Secretion of Enzymes 385
  E. Digestion of Special Kinds of Food 385
  F. Extraintestinal Digestion 388
  G. Reduction of Alimentary Canal and Enzymes 389
  H. Symbiosis 389
V. Carnivorous Plants 390
VI. Concluding Remarks 390
  References 392

I. Introduction

The food of animals consists mostly of reserve material or structural material of other animals or of plants. In the synthesis of these materials the greater part of the most active groups is "masked" by the formation of strong linkages, and polymeric substances of great chain length are formed which are chemically inert. They are either insoluble in water or, if soluble, only so in the colloidal state. Such substances are unable to pass through the walls of the gut and therefore must be reduced in molecular size. This reduction is performed in the process of digestion. For substances of lower molecular weight (as salts, cholesterol, vitamins), decrease in size is not necessary.

After the reduction in size of the molecules the process of absorption must be considered, as molecular weight is not the sole factor which determines the possibility of passage through the intestinal wall.

All digestion in animals consists of hydrolysis performed by means of enzymes, although often with the aid of various mechanical and nonenzymatic chemical devices [see references (*1*), Chapters 6 and 7; (*2*), Chapter 9; (*3*), Chapters 8, 18, 29]. These hydrolyses take place with little loss of energy. They are carried out in the intestine (digestive tract), where conditions of pH, temperature, etc., can be regulated to a certain extent but for the most part are not nearly as constant as in the "*milieu intérieur.*" Therefore the term "*milieu intermédiaire*" would be suitable to indicate the properties of this medium (cf. reference *4*, p. 89).

In this review the salient problems of digestion will be treated from a comparative point of view and the digestion of some special systematic groups will be described if these groups are scientifically or economically important or if the extent of our knowledge makes this appropriate.

From the extensive literature a choice had to be made. Preference in citation has been given to some authoritative handbooks, to reviews, to the most recent literature, from which the older literature can be found, and finally to some older references of fundamental importance.

II. Digestion in General

A. SECRETION OF ENZYMES (MAMMALS)

This subject is physiological rather than biochemical in nature and is extensively treated in many textbooks of physiology (*5–8*) and special works (*9, 10, 10a*) to which we may refer.

Enzymes are built up in the cells of the digestive glands or the digestive tract and stored in them mostly as "zymogen granula." If the gland proceeds to secretion, these granules are dissolved and secreted (merocrine secretion) or the whole cell is destroyed and the granula are liberated as such and dissolved in the gut (holocrine secretion) (*10*).

The secretion is regulated by nervous influences (*5–8, 12, 13*) and by the hormones of the intestinal canal (*1, 11–13a*) (see also Ancher, Chapter 6 in this volume). It is not the aim of this chapter to give a detailed description of all the digestive enzymes. For literature on these enzymes see the introduction of Section III.

B. ABSORPTION OF PRODUCTS OF DIGESTION

1. *Absorption in Vertebrates*

In vertebrates (mammals have most widely been studied) absorption of foodstuffs and water is localized chiefly in the small intestine. The surface of the absorbing intestine (especially in vertebrates) is enlarged by the presence of many folds. In the Tetrapoda (vertebrates with the exception of fishes) the surface is moreover enormously increased by the presence of the intestinal villi. These villi are small orifices at the inside of the gut about 1 mm. in length and 0.5 mm. in diameter, into which a lymph vessel, blood capillaries, and nerve elements penetrate. They are able to contract by means of smooth muscles (*14, 14a,b*). Recently it has been found by electron-microscopy that the luminal border of the cells of the villi (the "brush" border of previous authors) is lined with fingerlike projections, called microvilli, of about 1 μ in length and 0.1 μ in diameter. The enlargement of the surface by means of the folds of Kerkring (or valvulae conniventes), the villi, and the microvilli is respectively 3, 10, and 20 times, so that in all an increase of 600 times of the intestinal surface is reached (*14a*).

Absorption in vertebrates is a polar process, i.e., the passage takes place from the intestinal lumen (mucosal side) to the blood (serosal side of the gut). This phenomenon had been observed already by Reid (*15, 16*) in the course of his experiments with fragments of mucosa which separated salt solutions of equal composition. Moreover intestinal loops of the frog, filled under narcosis with isotonic or hypotonic solutions of glucose and replaced *in situ* are completely emptied in 2–3 days (*17*). Some subsequent objections to Reid's results (*18*) have been proved to have rested on the use of faulty techniques (*19, 20*); thus the polarity of the transport through the intestinal wall is firmly established—the more so as it takes place with consumption of oxygen (*21, 22*) and all later investigators have confirmed that it stops under anaerobic conditions.

The most conclusive results have been obtained with monosaccharides, these being neutral nonionizable substances. It has been proved decisively that glucose and galactose, the most important natural sugars taken up in the intestine, can be absorbed against a concentration gradient, or from isotonic solution (*23–27*). The velocity of this "active" absorption diminishes considerably in a caudal direction (*25*). Hexoses (mannose, sorbose) and pentoses (xylose, arabinose), which are not important as nutrients, penetrate the gut far more slowly and the velocity of their passage does not decrease in a caudal direction (25). Fructose occupies an intermediate position. Cori (*28, 29*), who studied these phenomena *in vivo* in rats by means of tube feeding without narcosis (the most natural way), obtained the ratios indicated in Table I (expressed in per cent of the value for glucose). Other authors (including Hele, whose results are also given in Table I) have found similar ratios (*30–32*).

TABLE I
Rates of Absorption and of Phosphorylation of Monosaccharides in the Rat[a]

Monosaccharide	Rate of absorption		Rate of phosphorylation
	Cori	Hele	Hele
D-Galactose	110	126	112
D-Glucose	100	100	100
D-Fructose	43	55	52
D-Mannose	19	36	25
L-Xylose	15	24	26
L-Arabinose	9	—	—

[a] Glucose is arbitrarily assumed to be 100. Results of Cori (*28*) and Hele (*32*).

As the absorption of glucose and galactose is inhibited by iodoacetate (*14*), Verzár and his co-workers tried to explain these results by the hypothesis that glucose and galactose undergo phosphorylation, by which process a concentration gradient from mucosa to serosa would be maintained. The argument was not a strong one, first because the concentrations of iodoacetate were so large as to be injurious to the animals [Höber (*33*), pp. 548, 549] and secondly because it was found soon afterward that it is not phosphorylation which is inhibited by iodoacetate, but other reactions in the sequence of glycolysis [cf. Baldwin (*2*), pp. 387, 415]. Nevertheless phosphorylation probably plays some part in the process of sugar absorption, as extracts of the mucosa are able to phosphorylate sugars. The rate of phosphorylation is correlated to the rate of absorption [Hele (*32*), cf. Table I]. In Hele's experiments mannose and pentoses are also phosphorylated to a certain extent. Anaerobiosis decreases the absorption of glucose and galactose at least three times, whereas the passage of the pentoses and of sorbose and mannose is increased in the absence of oxygen.

Not all the sugar that disappears from the lumen reaches the serosal side; part is "utilized," i.e., may serve as fuel for the energy-yielding process. Inhibition of absorption by iodoacetate or phlorizin may now be looked upon as inhibition of the energy-yielding process (in which phosphorylations play their part), but not especially as inhibition of phosphorylation.

The absorption velocity of fructose is intermediate between that of the easily absorbed and that of the slowly absorbed sugars (cf. Table I). During its passage it is converted to glucose. Anaerobiosis stops this conversion, but the diffusion of fructose is not stopped and proceeds even faster (*26*). The absorption of sugars has been reviewed by Crane (*33a*).

For lower fatty acids (C_2–C_6) it has also been found that they are transported from the lumen to the serosal side if the initial concentrations at both sides are equal (20.3 mmoles). As in the course of the experiment fatty acid disappears from the lumen and increases at the serosal side, transport against a concentration gradient is possible (*34*). At concentrations higher than 60 mmoles transfer is reduced, a finding which points to a transport mechanism that may become saturated. For absorption of higher fatty acids and fats, the presence of bile acids is necessary [cf. Haslewood (*35, 36*)]. By their emulsifying action they support the digestion of fat and are able to clarify emulsions of fatty acids (*14*) which they can bring into solution [probably colloidal (*37, 38, 38a*)]. Moreover, according to Frazer (*39–41*), monoglycerides are formed, which together with bile salts are powerful emulsifiers, so that part of the fat can be absorbed as such in the form of a very fine emulsion. (For emulsifiers in invertebrates, see Section IV,C.)

Up to 1950 not much evidence for active transport of amino acids had been produced, but since then many facts have been found in support of an active process. According to Fridhandler and Quastel (*42*) the passage of the L-forms of some of them is active, whereas that of the D-forms would be a diffusion. Anaerobiosis and dinitrophenol diminish absorption, but phlorizin has no such effect. Dipeptides are hardly absorbed as such; they appear at the serosal side as amino acids. Their hydrolysis may take place partly intracellularly (*43*).

Wiseman and co-workers (*44–50*) investigated extensively the transport of amino acids *in vivo* and *in vitro*. They found active transport against a concentration gradient for L-monoaminomonocarboxylic acids (exception: tryptophan), but not for glutamic and aspartic acids (rat, dog, cat, hamster). The velocity sequence from high to low (all L) is: proline, threonine, alanine, glycine, serine, valine, histidine, hydroxyproline, phenylalanine, isoleucine, methionine (*45, 46*). Pyridoxal has no stimulating influence on the uptake of these amino acids (*47*). If L-glutamic acid and L-aspartic acid are supplied in moderate concentration they are not transported as such, but are used in a transamination process in the wall of the gut which causes an increase in the concentration of alanine at the serosal side (*48, 49*). There is some competition among the amino acids, those which are more slowly absorbed inhibiting the absorption of the others if a mixture is given.

Whereas in the *in vitro* experiments of Wiseman isolated pouches of the intestine were used, Ballien and Schoffeniels (*51*) employed a technique in which the isolated mucosa was mounted between two frames of Plexiglas which separated two chambers, each filled with physiological solution to which the amino acids could be added. By means of calomel electrodes, the potential difference between the mucosal (negative) and serosal (positive) side could be measured (2–4 mv. for the small intestine, 20–50 mv. for the large intestine). This technique has the advantage that the muscular layer of the gut is absent, that the potential difference may be compensated if desired, and that the exposed surfaces can be measured. Outflux from and influx to the serosal side can be determined separately by means of labeled amino acids. In the small intestine of tortoise and guinea pig the influx for glycine, L-alanine, and L-histidine is of the order of 0.11 μmole/cm.^{2}/hr., the outflux of 0.01 μmole, so that the net influx is 0.1 μmole/cm.^{2}/hr. For the large intestine the influx is 0.22 μmole and the outflux 0.005 μmole, giving a net influx of 0.215 μmole [all values per square centimeter and per hour (*52*)]. (It is remarkable that the colon is able to play such an important part in the absorption of digestive products, as up to the present its role was considered to be limited chiefly to the absorption of water.) Compensating the potential difference does not abolish active transport; therefore the existing potential difference is not the cause of the transfer of amino acids. As the net influx is decreased by addition of dinitrophenol, metabolic processes are probably responsible for the active transport [see reference *53;* cf. also (*54*), Chapter 17]. The much larger potential difference between serosa and mucosa of the colon as compared to that of the small intestine may be explained by differences in permeability of the membranes for Na$^+$ and K$^+$, resulting in the formation of a much more explicit double layer at the outer (+) and inner (−) surfaces of the colon than at those of the small intestine (*53*).

That an isotonic saline can be taken up is known already from the results of Reid (*15, 16*). On the question whether the absorption of water is an active or passive process opinions are divided. The first view is held by Visscher *et al.* (*55*), Parsons *et al.* (*27*), and Fisher (*57*). The absorption of water is decreased by phlorizin if glucose is present, but not if it is absent. The presence of glucose facilitates uptake of water (*34*). The second view is advocated by Jervis *et al.* (*56*) and by Curran and Solomon (58), who give an elaborate discussion of the problem. According to them, water moves in accordance with osmotic pressure and, if inner and outer concentrations are equal, is dependent on the net solute flux. However, the transport of Na^+ and Cl^- is active. Besides the mechanisms mentioned above, the contraction of the villi plays a part in the mechanism of absorption. But in a great number of vertebrates having villi such movements have not been observed (*14, 14a*).

The problem of absorption is being much investigated at present; full particulars for mammals can be found in some recent reviews (*59, 60*), and for an evaluation of the successive theories of absorption Verzár and McDougall (*14*), Höber (*33*), Wilson (*14a*), and reference *14b* may be consulted.

Quite recently Miller and Crane (*60a*) proved that the enzymes which hydrolyze disaccharides are not secreted into the small intestine, but are working within the microvilli, which could be isolated (compare reference *42* for the similar conclusion for dipeptides). The small enzymatic activity found in the succus entericus (intestinal juice) originates from squamated cell fragments. Newey *et al.* (*60b*) arrived at similar results. They found maltase activity restricted to the brush border (microvilli). Inside this zone is a second zone where a phlorrhizin sensitive glucose-transferring mechanism is located, and a third zone where glucose is metabolized and where a fluid transfer mechanism functions.

Barry and co-workers (*60c*) corrected the previous results (*25*) that the intensity of the absorption of glucose decreases in the caudal direction. They found the greatest capacity for transfer of glucose and water *together* in the middle section of the intestine. In the lower intestine there is a (smaller) glucose-independent transfer of water. The energy for the first mechanism would originate from glycolysis; that for the second mechanism from the citric acid cycle. However, Curran (*60d*) maintains that water transport is not possible unless there is a "net" solute movement. In order to elucidate the controversial evidence on active or passive transport of water he designed a model for which the original paper should be consulted. Moreover he found that the flux of Na^+ and Cl^- *in vitro* is considerably lower than *in vivo*. The transport of these ions is not dependent on the existence of an electric potential gradient between the mucosal and serosal side of the mucosa. This result agrees with that of Ballien and Schoffeniels (*51–54*) for amino acids. The transport of NaCl does not take place in the absence of glucose. Thus the dependency of the transport of water on that of NaCl is indirect.

Experiments on absorption of sugars in some lower vertebrates have been recently carried out by Cordier and co-workers (*61*), here also references on their earlier work. Their results on the velocity of absorption partly agree with, partly deviate from, those with mammals (Table II). The slow absorption of fructose in pike and *Scorpaena* (both carnivores) is ascribed by the authors to the lack of stereokinase which can transfer fructose to glucose. The pH optimum of glucose absorption coincides with the pH of the gut (9.0 in *Scorpaena,* 8.4 in *Tinca,* 8.3 in *Rana*). With increase in temperature, absorption of glucose and galactose increases per 10° C. by a factor 1.4–2.0 (Q_{10}), according to the rule of Van't Hoff-Arrhenius (*Tinca, Rana*); for the passively absorbed pentoses the increase is smaller. Ivanov (*62*) has found that extirpation of the cerebral hemispheres in the frog depresses absorption of glucose by 50%. The absorption of water is also decreased, though less strongly. After 2 hours, in normal animals 3–3.5 ml. of the original 5 ml. of water has been absorbed; after extirpation, 2–2.5 ml. These numbers confirm again the polarity of the process in lower vertebrates (cf. reference *17*).

TABLE II

ABSORPTION VELOCITY OF MONOSACCHARIDES IN LOWER VERTEBRATES[a]

Pike (*Esox lucius*)	galactose > glucose > arabinose > xylose > fructose
Scorpaena porcus	galactose > glucose > arabinose > xylose > fructose
Tench (*Tinca vulgaris*)	galactose ≥ glucose > fructose > xylose
Frog (*Rana esculenta*)	galactose > glucose > fructose > xylose > arabinose
Rat	galactose > glucose > fructose > xylose > arabinose

[a] After results of Cordier and Maurice (*61*).

2. *Absorption in Invertebrates*

Invertebrates show more variability in the type of absorption than vertebrates. In view of the extremely large anatomical differences between the various types of invertebrates, only a very small number of their representatives have been studied.

In *Helix* the intestine behaves as a diffusion membrane, permeable to water and solutes. If the gut is filled *in vivo* or *in vitro* with solutions of glucose or sodium chloride, sugar or salt are exchanged by diffusion, but the amount of fluid in midgut, crop, or rectum at the end of the experiment is the same as at the beginning, whereas in *Rana* the intestine is totally emptied (*63*).

In *Holothuria* it has been found [Enriques (*64*), Oomen (*65*)] that the intestine is semipermeable: glucose, NaCl, urea do not pass, whereas water passes if an osmotic gradient is present, but the gut is never emptied. These investigations corrected the results of Cohnheim (*66*), who, using a faulty technique, thought he had found a true absorption with emptying of the gut, as in vertebrates. Nevertheless, the semipermeability of this gut is astonishing as it does not seem to be chitinized and there are no other absorbing surfaces. Absorption seems to take place here by means of migrating cells which accumulate in the wall of the gut during digestion and have been shown to take up dyes from the intestine (*65*). [Collaboration of such cells in absorption has been found also in the oyster (*67*).] However, in recent work with C^{14}-labeled food (*65a*) permeation through the intestinal wall in a holothurian was found, although the absorbed material is mainly stored in the wall of the gut.

In higher crustaceans foregut and hindgut are lined with chitin and behave like semipermeable membranes [*Astacus* (*17*), *Nephrops* (*68*), and *Homarus* (*63, 69*)]. For *Nephrops* absorption of fat in the midgut has been proved (*68*). However the midgut in Decapoda is often short. Probably the chief organ for absorption there is the hepatopancreas. In *Nephrops* (*68*) the uptake of iron (as lactate or saccharate) in the absorption cells of this organ could be demonstrated; in *Atya* the absorption also of fat (*70*); in *Hemigrapsus,* that of phosphate-P^{32} (*71*). In mollusks the hepatopancreas also plays an important part in absorption, but in lamellibranchs and various gastropods its demonstration is complicated by the fact that, in this organ, phagocytosis also takes place (for phagocytosis see Section IV,A). However, as a result of feeding iron lactate or saccharate absorption has been been found in this organ in *Murex* (*72*) and *Ostrea* (*67*).

The most elaborate recent studies on invertebrate absorption have been carried out with insects. Absorption of glucose in *Schistocerca* (locust) and *Periplaneta* (roach) takes place chiefly in the midgut. The glucose taken up appears in the hemolymph as trehalose (*73*). Thus, as disaccharides do not pass rapidly through the intestinal wall, a concentration gradient of glucose is maintained. To a certain extent this can be considered to be an "active" process, though of another kind than that in mammals ("facilitated diffusion"). Similar facts have been found for a blowfly (*74*). In *Periplaneta,* fructose is converted to glucose before absorption (*75*). Moreover, in various insects there are differences in the absorption velocity of different sugars (*75*). In *Drosophila* fructose would be absorbed more rapidly than glucose (*76*). The absorption of amino acids [locust (*77*)] is slower than that of the mentioned hexoses. The extremely high concentration of amino acids in insect hemolymph [of the order of 100–300 mg. per 100 ml., against 30–40 mg. per 100 ml. in the blood of mammals (*79*)], must be an obstacle to absorption. Absorption of the amino acids is preceded by absorption of water, which causes a gradient from the lumen of the gut to the hemolymph, after which exchange of glycine and serine takes place (determined by means of C^{14}-labeled compounds). Further investigation seems desirable.

The absorption of fats (tripalmitin and palmitic acid) takes place chiefly in the anterior part of the midgut. Digestion of fats (in *Periplaneta*) is a slow process (*78*).

Whether absorption of water and salts in insects is an active or a passive process cannot yet be decided. As we saw, even for mammals it is still debatable whether water is passively transported by salts or glucose, or actively absorbed.

C. Role of Intestinal Movements

The movements of the intestine, fully treated in textbooks of physiology and anatomy (*80–83a*), must play an important part in digestion, although the investigation of this effect has been rather neglected. First, they sustain considerably the action of the enzymes. In experiments *in vitro* it has been found (see reference *84*, pp. 189, 190) that even very moderate stirring (50–75 r.p.m.) of fibrin grains in a solution of trypsin increases digestion by 100% to 150%. Qualitatively, a similar effect has been observed for pepsin (*84*). The so-called segmentations and pendulum movements must have the same effect *in vivo*, and similarly the strong contractions of the gizzard in birds and devices like the gastric mill in crustaceans must have this effect. Moreover, digestion is enhanced by the mixing of enzymes with the food by these movements, which also continuously bring fresh portions of digestion products in contact with the wall of the gut (*80, 81*). Without this last aid continuous absorption of digestive products would hardly be possible. This continuous withdrawal of splitting products must in its turn accelerate the cleavage process (*84a*).

III. Digestion in Special Physiological and Systematic Groups of Vertebrates

The properties of carbohydrases have been treated elsewhere in this treatise by Bernfeld (Volume III, Chapter 8). For those of exo- and endoproteinases and lipases the works of Boyer, Lardy, and Myrbäck (*86b*), Bersin (*86c*), Barrington (*86d*), and references *1–3*, *13*, *85*, and *88* may be consulted.* (References *13*, *85*, and *86d* are written from the comparative point of view.) The general aspects of secretion, absorption, and intestinal movements, were described in the preceding pages. Thus we have to consider here chiefly the collaboration of these various factors in the digestive process. Detailed information is to be found in references

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* See also Taylor (*93a*) and Herriott (*93b*).

1, 4, 5, 6, 8, 10, 12, 13, 87; the "Tabulae Biologicae" (*87*) contain many numerical data.

A. Role of the Mouth of Vertebrates in Digestion

The part played by the mouth in the chemical digestive process is very limited.* The saliva of mammals contains fair amounts of amylase only in man, in primates, and (less) in Rodentia and Suidae (see reference *85*, p. 269). Recently it has been found (*86*) that the saliva of dogs on a diet rich in carbohydrate contains a small amount of amylase which disappears after the animals are placed on a diet of meat.† If amylase is present in saliva, the enzyme can act in the stomach, but only so long as the stomach contents have not reached a low pH. For action in the mouth the time is rather short.

The chief function of saliva is to furnish the glucoprotein mucin which facilitates deglutition.

B. Stomach and Intestine of Monogastric Vertebrates

The gastric juice contains endoproteinases with pH optima at 1.5–2.0 and 3.0–3.5 (Fig. 1). The enzyme with the lowest optimum has long been known as pepsin (*88*). It has been a much debated question whether the optimum at 3.5 is that for a separate enzyme comparable to the cathepsin of internal organs, or whether there is one enzyme with two optima [Buchs (*89, 90, 90a–c*); Taylor (*91–93a*); Richmond *et al.* (*94*); Tang *et al.* (*94a*); Geilenkirchen *et al.* (*95*); Merten *et al.* (*96*); Miller *et al.* (*97*); Crebolder *et al.* (*98*)].

The recent elaborate work of Taylor (*91–93a*) led to the conclusion that in man and swine two endoproteinases are secreted by the stomach wall, one by the fundus, the other by the pyloric part. Each of these has two optima on certain substrates, but these optima differ slightly for fundus and pyloric part (Fig. 1). Both in man and pig the two pyloric pH optima are somewhat lower than the two fundal pH optima. Crystalline pepsin contains two enzymatic components, the optima of which correspond rather closely to those of the enzymes isolated from fundus and pyloric part. Thus, according to Taylor these two enzymes are both pepsins, the activities of each enzyme at low and higher pH cannot be separated from each other, and the higher optimum does not point to a catheptic activity. By certain modes of extraction the higher optima may be suppressed. Moreover on some substrates (egg albumin) only one

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* Chewing (which occurs in many animals), being a purely physiological act is not treated here, but it must be mentioned as a preparation to digestion.

† For a discussion of this so-called "enzymatic adaptation" see a review by Knox *et al.* (*86a*) and Ben Abdeljlil *et al.* (Addendum).

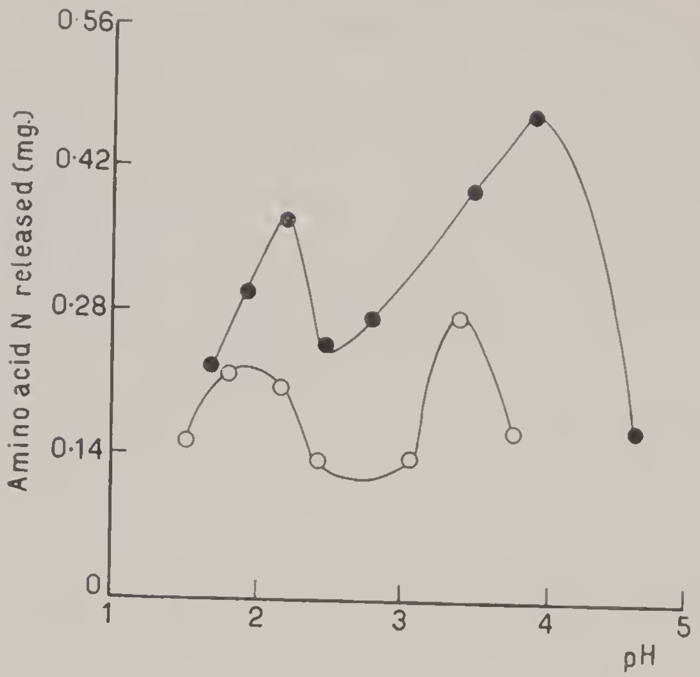

FIG. 1. pH-activity curve for the digestion of bovine plasma albumin (fraction V) by fundic (●) and pyloric (○) mucosal extracts of the pig. Temperature 37°; time 3 hours. After Taylor (*91*, p. 386).

optimum is found. These complications explain the many contradictory results of previous investigators. However, Richmond *et al.* (*94*), Tang *et al.* (*94a*), and Ryle and Porter (*94b*) reported the separation of two pepsin components: one with an optimum at approximately pH 2, the other at pH 3.5, so that the question is not yet finally decided. It seems unlikely that the latter enzyme is identical with cathepsin, as Buchs (*89*) originally claimed. Ryle and Porter (*94b*) found two enzymes in pyloric extracts, which they called parapepsin I and II. However, the fact that there are two optima is more important biologically than the question whether these are caused by one enzyme or by two or three.

Moreover, a stomach lipase is present (optimum 4.5–5.0) (acting only on emulsified fat) and a milk clotting enzyme (rennin or chymosin, optimum 6–7), the latter chiefly in young mammals. As the pH of the gastric juice is 0.8–1.6 (*87*), the important question arises how enzymes with such different optima can work together in the stomach (*4, 109*). Obviously this would hardly be possible if gastric juice and contents were uniformly mixed so that a uniform pH throughout the whole stomach resulted. It has long been known that this is usually not the case. Experiments in which animals were fed different kinds of food or differently colored foods, and subsequently killed, have revealed that the contents, taken up successively, remain in their original relative positions as long as they are not influenced by digestion (e.g., *99, 100, 101, 101a*). This was confirmed for the human stomach by means of X-ray examination [Groedel; see Klee (*102*)]. Moreover, it has been shown that milk which forms a clot in the mammalian stomach shows only an acid reaction in the marginal zone (if the milk has been colored by indicators before ingestion) [Tobler (*103*)]. Later it was proved by measurements with the glass electrode that, in animals like the pike and snakes which take up their prey as a whole, only in the border zone of this prey is there a reaction favorable for peptic digestion [Mennega (*104*); Vonk (*4*)]. In carnivores that rend their prey or take up several small animals, at first a pH proper for digestion is reached only in the outer layer of the food, but later a suitable pH is reached also in the interior of the stomach (*4, 104*). In dogfishes, which have a very strongly acid secretion, a pH of 2.3–3.2 suitable for digestion is soon reached throughout the stomach (*105*).

However, some difficulties remain to be resolved. From elaborate experiments in which the pH was measured after regular time intervals at definite places on the surface of the stomach contents of the rat, it could be concluded that one-third of the contents had left the stomach before a pH of 3.5 had been reached, and 85% before their surface had reached pH 2.0 [van den Broek and de Groot (*106*)]. For human digestion, similar results have recently been obtained by the intubation method [Borgström *et al.* (*107*)]. Moreover, in fish-eating birds like the heron, the surface of the contents hardly reached a pH lower than 4.0 (*4, 104*). These difficulties could be resolved when the presence of a second optimum of about 3.5 had been firmly established [Buchs (*89–90c*); Taylor (*91*)]. Thus it is not surprising that in some animals the pH hardly is lower than 4 (birds) and that in mammals a great deal of the food has left the stomach before pH values of 2–3 are attained. In these cases the greater part of the digestion must have been performed by the action of the proteinase which has its optimum at 3.5–4.0.

With these considerations in mind, the question can also be answered how a lipase with an optimum pH of 4–5 can act in the stomach. (This enzyme is active only on emulsified fats like those of milk and egg yolk.) However, the optimum of rennin (pH 6.0–7.0) is very different from the pH values of 2–4 which may be reached in the stomach. It develops its action at the beginning of the digestive process. Now it is a fact that in young mammals the gastric juice is less acid than that of adults (for pertinent literature, see references *4* and *109*). Moreover, there is some evidence that in young mammals conditioned reflexes are not yet or not fully developed (*108*). These two circumstances, combined with the buffering action of the milk, make it probable that in young animals at the beginning of a meal the stomach contents will have a pH high enough for the action of rennin. Freudenberg (*109*) has given attention to this interplay of actions in the stomach in a monograph (summarized in reference *4*).

It is still an open question how the gastric juice can act on substances like vegetables and starch which are not attacked by its enzymes. Apparently it has some such action, for the food of herbivores leaves the stomach as a more or less homogenous chyme. Moreover, in the older literature it is reported that in some animals (e.g., horse) lactic acid and proteolytic fermentations of food (but not of cellulose) take place in the cardiac gland zone (*101, 101a,b*). Where the acidity remains low the enzymes in the food may also collaborate (*101*). These points have not been reinvestigated in recent times.

These questions of integration of different actions mentioned above are hardly considered in textbooks of physiology and physiological chemistry. Recently Davenport (*60*) concluded in a review on digestion: "The physiological significance of the second enzyme having a pH-activity peak at 3.5 has not been determined. . . ." Such significance, as we have seen, can quite well be suggested if the facts known from comparative physiology are taken into consideration. Pepsin can then be looked upon as a versatile enzyme, or couple of enzymes, able to meet the exigencies of different conditions of pH in the stomach.

The question whether the enzymes are working at their optimal conditions can also be posed for the small intestine of vertebrates. Whereas older investigators accepted an alkaline reaction of the contents (because loss of CO_2 was not prevented in the measurements), we now know that the intestinal contents have a pH of 5–6 in carnivores, of 6–8 in omnivores, and of 7–9 in herbivores (summarized in references *4, 87*). In omnivores the carbohydrases are working more or less under optimal conditions; in the other groups there are deviations to both sides of the optima, but they are so slight that a fair action of these enzymes is possible. However, in carnivores the reaction of the chyme deviates by one or two points to the acid side from the optimum for the proteolytic enzymes. Several factors may contribute in compensating more or less for the unfavorable effect of this deviation. These are: (*a*) the movements of the gut, which enhance digestion appreciably (*84*); (*b*) continuous secretion of fresh enzymes; (*c*) continuous removal of cleavage products by means of absorption [Lea (*110*)]. For some substrates activation of proteolytic enzymes by means of bile acids has been supposed (*4, 84*), but this has also been partly contradicted (*111*).

As to the action of temperature on digestion, in mammals and birds the enzymes are working under conditions not very far from their optimum. The temperature of poikilothermic animals may vary considerably. Enzymes of several lower forms were found to be less resistant to temperature than comparable enzymes of mammals and birds (*112, 113*). But this is no general rule: even the reverse has sometimes been found.

The amylase of the Indian crab *Parathelphusa* (*114*) is less resistant than that of *Nephrops* living in Northern seas (*68*). The literature on this point up to 1940 has been reviewed in ref. *115*.

The point that animal amylases hardly attack intact starch grains, is mentioned in Section IV,C.

The stomach of some insectivorous mammals [*Rhinolophus ferrum equinum* (Chiroptera) and the insectivores *Erinaceus europaeus* and *Talpa europaea*] according to Jeuniaux (*116*) secretes chitinase, but no chitobiase, so that in the stomach digestion does not give rise to the formation of acetylglucosamine. In the cat and the rabbit the stomach does not secrete chitinase, but it has been found in the stomach of the blackbird and the sparrow (*116a*).

C. RUMINANTS AND ANIMALS WITH SIMILAR CHEMICAL MODES OF DIGESTION

The ruminants are a group of mammals that are beautifully adapted to the digestion of very resistant, cellulose-rich food. Systematically the group includes the families Camelidae, Tragulidae, Cervidae, Bovidae, and Giraffidae.* These animals are polygastric: the stomach is divided into four compartments: rumen, reticulum, omasum, and abomasum (Fig. 2). The rumen is by far the largest compartment (its volume is approximately 60 liters in the cow); the inner surface of the much smaller reticulum is covered with a network of folds. The omasum is provided internally with leaves like the pages of a book; the abomasum is

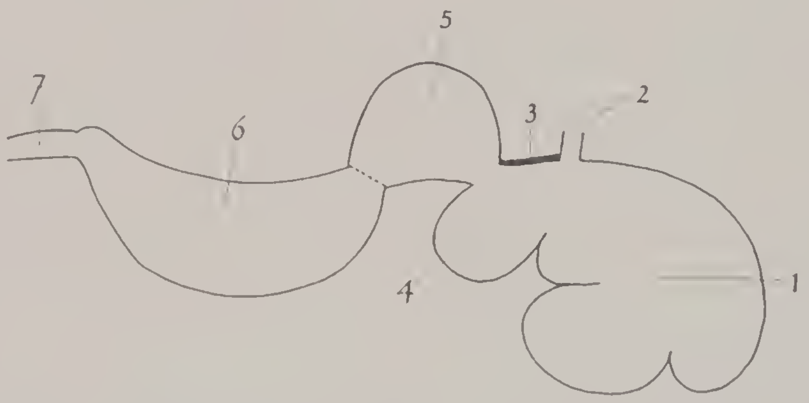

FIG. 2. Schematic diagram of the stomach compartments of cow and sheep. KEY: *1*, rumen; *2*, esophagus; *3*, esophageal groove (connection between the three first compartments); *4*, reticulum; *5*, omasum; *6*, abomasum; *7*, duodenum. After Mangold (*101*).

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* The success of this group in the evolution appears from the great number of genera, species and individuals from which it is composed.

comparable to the stomach of monogastric mammals and secretes the same enzymes and HCl (*6, 101*). For the anatomy see references *101* and *117*.

The food is taken up by the two first compartments and is subjected to fermentation under the influence of many species of bacteria. The chief products of this fermentation which can be used by the animal are: acetic, propionic, butyric, and lactic acids, the amounts decreasing in the order listed (*118–122*). The concentration of volatile acids in the blood draining the rumen is considerably higher than that in the peripheral blood and significantly higher in blood draining the omasum and cecum, but not so in vessels leaving the abomasum and the small intestine. This shows that the volatile acids are directly absorbed, chiefly by the mucosa of the rumen (*123*). For later work on the omasum see page 363. The surface of the rumen has deep grooves where its wall is very thin (*124*), and there this absorption may take place. These acids can be used in the metabolic processes for synthesis of fat, glycogen, and amino acids, and they can serve as fuel. For acetate it was shown by Barcroft (*125*) that this can replace glucose as fuel for a frog heart beating *in vitro*.

These fermentations lead also to the production of methane and carbon dioxide, which are removed by ructus. The approximate composition of the gas in the rumen is: CO_2, 65%; CH_4, 30–35%; N_2, 2–4%; $O_2 <$ 1%. Nitrogen and oxygen are supplanted by carbon dioxide and methane (*6, 101*). As methane still has a high combustion value, these fermentation processes are uneconomical from an energetic point of view, but this is compensated by the large amounts of food which can be taken up.

During the time that these fermentations are in progress the food mass is regurgitated several times to the mouth and ruminated.*

The bacteria that aid in digestion are able to build up their protein from the protein in the food of ruminants and also from simple nitrogen compounds like ammonium salts present in the plants (*126, 127*). On the bacteria subsists a fauna of protozoa. Both bacteria and protozoa are carried on to the abomasum and killed and digested there. The amount of protein thus formed in the rumen of the cow is estimated at 200–300 gm. daily and forms a considerable supplement to the low protein ration provided by grass, which is moreover partly decomposed by the bacteria (grass contains 1–2% protein in terms of fresh weight). Urea may also serve as a material for this protein synthesis. It is sometimes used as such in practice. For effective synthesis of protein from urea much starch must be present (*126*). The urea produced by the animal itself is excreted to a considerable extent in the saliva and thus may be used anew by the bacteria in protein synthesis (*126–129*). Chalmers and Synge (*126*) estimate, however, that the part played by nonprotein nitrogen in the synthesis of protein by the rumen bacteria has been exaggerated as compared to that of the pre-existing protein of the food. In the wild state nonprotein nitrogen would be important for this purpose only in winter and in periods of drought. By means of the rumen bacteria the animals are also able to incorporate inorganic sulfur in their sulfur-containing amino acids, a faculty which is lacking in nonruminants. This has been demonstrated by isotope experiments (*130, 131*).

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* During the sucking period the milk passes directly through the esophageal groove (3 in Fig. 2) from the esophagus into the omasum: the rumination mechanism is still inactive.

The bacteria have yet another useful function: they are able to synthesize almost all the vitamins of the B complex (B_1, B_2, B_6, niacin, pantothenic acid) so that vitamin B deficiencies seldom occur in ruminants (*118–120, 131a*).

In contrast to the stomach of monogastric animals, the movements of the rumen and reticulum of ruminants are excessive and the food mass is moved continuously from rumen to reticulum and vice versa; the movement is a mechanical aid to the bacterial fermentation process. In the omasum much water is lost by absorption (*132*), not, as was once thought, by pressure. Absorption of the lower fatty acids from rumen and omasum (see also page 362) has been restudied by Masson and Phillipson (*133*), Phillipson (*134*), and Pfander and Phillipson (*135*). The concentration of volatile fatty acids in the chyme leaving the abomasum is from seven to twenty times lower than that in the rumen, thus considerable absorption has taken place before the chyme reaches the small intestine. The movement of water, sodium, and potassium through the rumen epithelium was also studied (*136*). The transport of Cl^- against a concentration gradient (*137, 138*) has been attributed to a potential which makes the plasma of the sheep about 30 mv. positive to the contents of the rumen. It remains to be seen whether this view is consistent with the fact mentioned in references *51–54* (Section II,B) that the absorption of amino acids is not decreased if the potential between the mucosal (—) and serosal side (+) of the gut is compensated. If this potential were the driving force for absorption of Cl^- from the rumen, what then must be the driving force for the absorption of Na^+?

The composition of the bacterial flora in the rumen is of great importance for the efficiency of the process of rumination. If ruminants are kept for 8–14 days on a diet of bad quality hay, fermentation is poor and only small amounts of fatty acids are formed [Elsden (*139*)]. The characteristic species of bacteria necessary for a good fermentation are then replaced by other less effective forms. By supplying food of good quality the original status can be restored. Similarly the yield of milk and its percentage of fat of European cattle in the tropics could be considerably raised by keeping the animals on meadows with grass of good quality [Schoorl (*139a*)]. The species of bacteria necessary for good fermentation have been studied extensively (*140–142*). The species of Protozoa have been reviewed by Oxford (*142a*).

Much less is known of the digestion of wild living ruminants. The digestibility of the food of North American deer (*143*) and its basal metabolism (*144*) have been studied. If these animals browse in spring and summer, they prefer a variety of plants in their diet (*145*). Only bitter brush may be taken as the sole plant for prolonged periods (for additional literature, see references *143* and *144*).

Some results of quite recent work may still be added here. Ash (*150a*) has investigated the secretion of HCl in the abomasum (sheep). The secretion is evoked by distention of the abomasum, by introduction of rumen fluid and of buffered solutions at pH 5–7; it is reinforced by the presence of short-chain fatty acids in these fluids. Inhibition of secretion occurred when the free fluid in the abomasum had reached a pH of 2.0 or if HCl solutions of pH 2.1–2.4 were introduced. Fatty acid was absorbed from the solutions introduced into the abomasum. Magee (*150b*) studied the external secretion of the pancreas in the sheep. This, as well as the amount of amylase, is reduced 50% by fasting. Both are strongly increased by introduction into the duodenum of abomasal contents, or of a 55 mM fatty acid solution or 0.1 N HCl. Also by injection of secretin and pilocarpin. Distension of the abomasum was without effect (it has such an effect in dogs). The pH of the duodenal contents in fed animals was remarkably low, i.e., never above 3. The abomasal contents entering the duodenum have the same value and are apparently not neutralized before they are well beyond the common opening of the pancreatic and bile ducts. This low pH must, according to the author, play an important part in the natural evocation of the secretion.

By inserting cannulae into the duodenum of the sheep Harris and Phillipson (*150c*) established that the quantities of organic matter that disappear in the stomach represent half or more of the total amount that disappears in the digestive tract. Substantially more N left the abomasum than was taken up daily in the food chiefly due to transfer of blood urea to the rumen. Of the N, passing the duodenum, the greater part was absorbed in the intestine. About 400 ml. of abomasal contents passed the duodenum per hour. Special experiments (in which chromium sesquioxide was administered) proved that the conditions of passage were normal.

Phillipson *et al.* (*150d*) reinvestigated in the sheep the faculty of using ammonia for the synthesis of protein by the rumen bacteria which are able to ferment starch and glucose. [It was already known that in the rumen urea is rapidly hydrolyzed to ammonia (*150e*).] On a diet with much flaked maize, the concentration of previously added ammonia (as NH_4Cl) decreased. This was accompanied by an increase in compounds precipitated by trichloroacetic acid. If N^{15}-labeled ammonium chloride was used, the N^{15} was concentrated in this precipitate. The isolated starch- and glucose-fermenting bacteria produced acetic and lactic acids and could also use L-cystine, B-vitamins, and (less rapidly) peptones as the nitrogen source. These actions of the bacteria *in vitro* were not decreased by the addition of sulfanilamide, penicillin, or chlortetracycline.

For an introduction to the problems and the literature on ruminants, references *118*, *118a*, *126*, and *146–149* may be consulted. Numerical data are to be found in references *87* and *150*. The act of rumination proper is treated in references *147* and *149*. For an exhaustive treatment of the physiological and biochemical processes of rumination three recent books (*150f,g,h*) may be consulted.

All in all, the digestive process in ruminants is a high biological

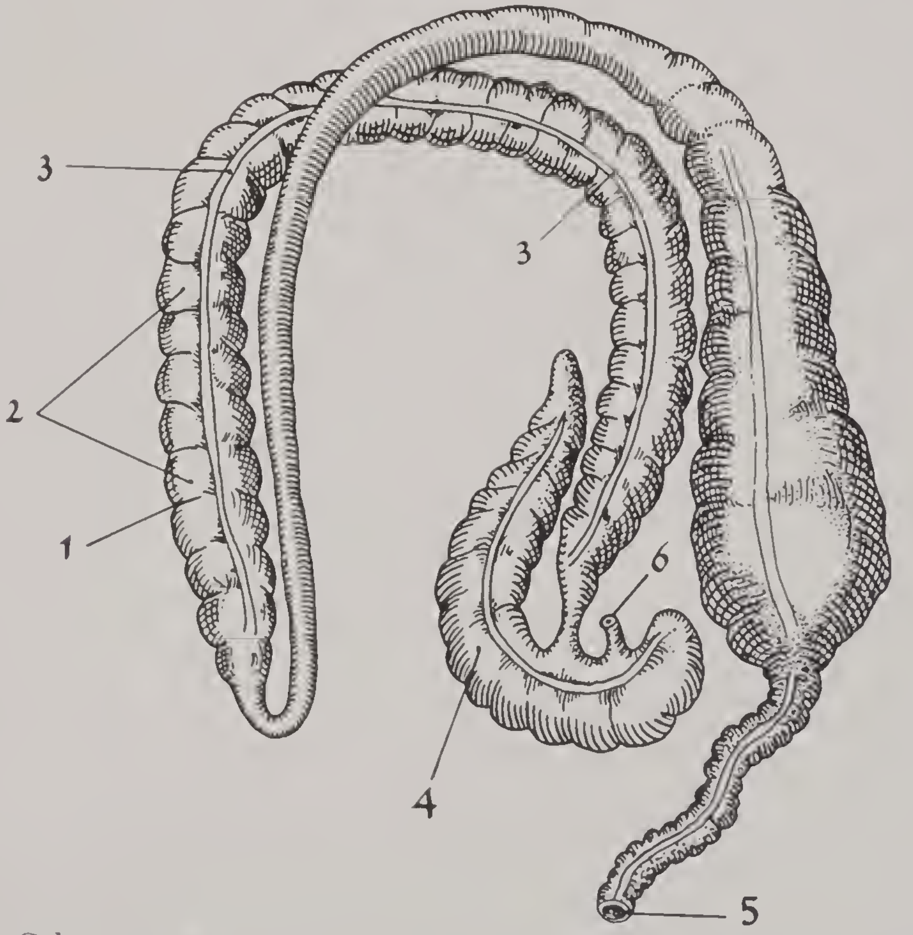

FIG. 3. Colon and cecum of the horse. KEY: *1*, Plica; *2*, haustra of ventral colon; *3*, taenia; *4*, cecum, also with haustra; *5*, small colon; *6*, small intestine. Mark the strong development of both cecum and colon, the latter, unlike that of man, having a ventral and a dorsal part. After Ellenberger and Baum, from Mangold (*101*).

adaptation which is equally important biologically and in agricultural practice.

The various peculiarities treated here for ruminants (though less extensively studied) are present also in herbivores, which possess a strongly developed cecum and colon instead of the polygastric stomach. Examples of such animals are the horse (Fig. 3), the pig, and the rabbit. In their large cecum and colon the same processes, like e.g., formation of lower fatty acids and synthesis of B vitamins, take place and the products are absorbed (*118–120*). These findings have been confirmed for the rabbit by Cools and Jeuniaux (*151*). Cellulose is partly digested in the cecum, probably by bacteria. At the height of digestion the concentration of fatty acids in the blood of the mesenteric anterior vein is 8–10 times higher than in the blood of the mesenteric and carotid arteries.

Some Marsupialia have essentially the characteristics of ruminants [Moir *et al.* (*152, 152a*)] but are without the proper rumination movements. *Setonyx* ("quokka" or wallaby) has a large sacculated "forestomach" in which a distinct esophageal groove is present (Fig. 4). In this forestomach extensive bacterial fermentation leads to the formation

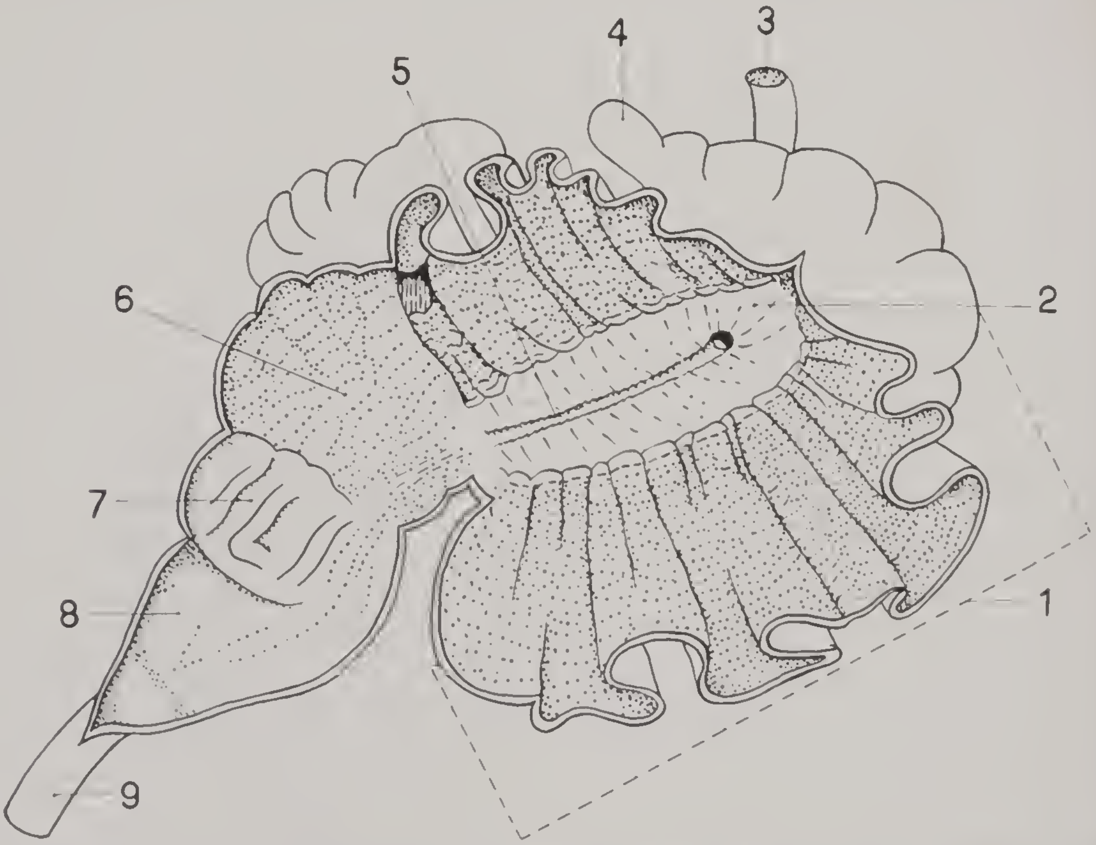

FIG. 4. Anatomy of the stomach of *Setonyx* (Marsupialia). KEY: *1*, putative rumen (sacculated area); *2*, stratified squamous epithelium; *3*, esophagus; *4*, cul de sac; *5*, esophageal groove; *6*, nonsacculated area; *7*, like *6*, area of unknown significance; *8*, fundus (highly acidic and analogous to the abomasum of ruminants). After Moir *et al.* (*152a*).

of volatile fatty acids, CO_2 and CH_4 as in ruminants. Protozoa have not been found. The much lower concentration of volatile fatty acids in the fundus points to their absorption in the sacculated part of the stomach. As in ruminants, blood sugar values are much lower than in other mammals, not much glucose reaching the small intestine. Related forms have the same peculiarities as to the anatomy of the stomach and the occurrence of a large bacterial flora in the forestomach. According to Calaby (*153*), the digestibility of crude fiber in *Setonyx* is 25–48% [less than in ruminants (70–80%), but better than in the rabbit]. The passage of the food in *Setonyx* is more rapid than in the ruminants. As the Marsupialia are not closely related to the other mammals, the development of a rumination process in some marsupials may be considered as a case of convergent evolution (152a). Another case of such convergent development is, that the stomach of the sloth (*Choloepus hoffmanni* Pet.) is enlarged by pouches in which bacterial fermentation of the strictly herbivorous food takes place. As in *Setonyx* there is an esophageal groove, but no rumination movements have been observed [Jeuniaux (*153a*)]. Thus it seems that the occurrence of a bacterial fermentation process in the stomach is the most essential stage in these convergent developments and that the true ruminants have completed this process by acquiring the faculty of rumination movements.

D. BIRDS

Birds are another type of polygastric animal, their stomach being in most forms differentiated into a proventriculus (or glandular stomach) and a gizzard or ventriculus. Moreover most birds have a crop in which the food is collected and from which it moves in small portions through the esophagus to the proventriculus. The latter produces pepsin and HCl; it is a small organ and its walls are thin in comparison to those of the gizzard. The wall of the gizzard is composed of very strong muscle layers, in some places reinforced by sinew plates, so that in longitudinal section it looks like a very heavy-walled tube (Fig. 5). The inside is lined with so-called coilin, a kind of keratin, as a protective layer. The rest of the intestine is rather undifferentiated; the large intestine (colon) is not very different in aspect from the small intestine; contrary to the situation in mammals there are no haustra. There are often two ceca; their size differs in the various species [*101*, *117*, *154*, *154a*; also (*6*), Chapter 17].

The most peculiar part of the intestine is the gizzard, which exhibits rhythmic contractions (frequency in the chick 2.5 per minute in hunger, 3 per minute if food is present) (*155*, *156*). The great pressure which the organ can exert was noted already by the first investigators of avian digestion (Redi, Réaumur, Spallanzani).

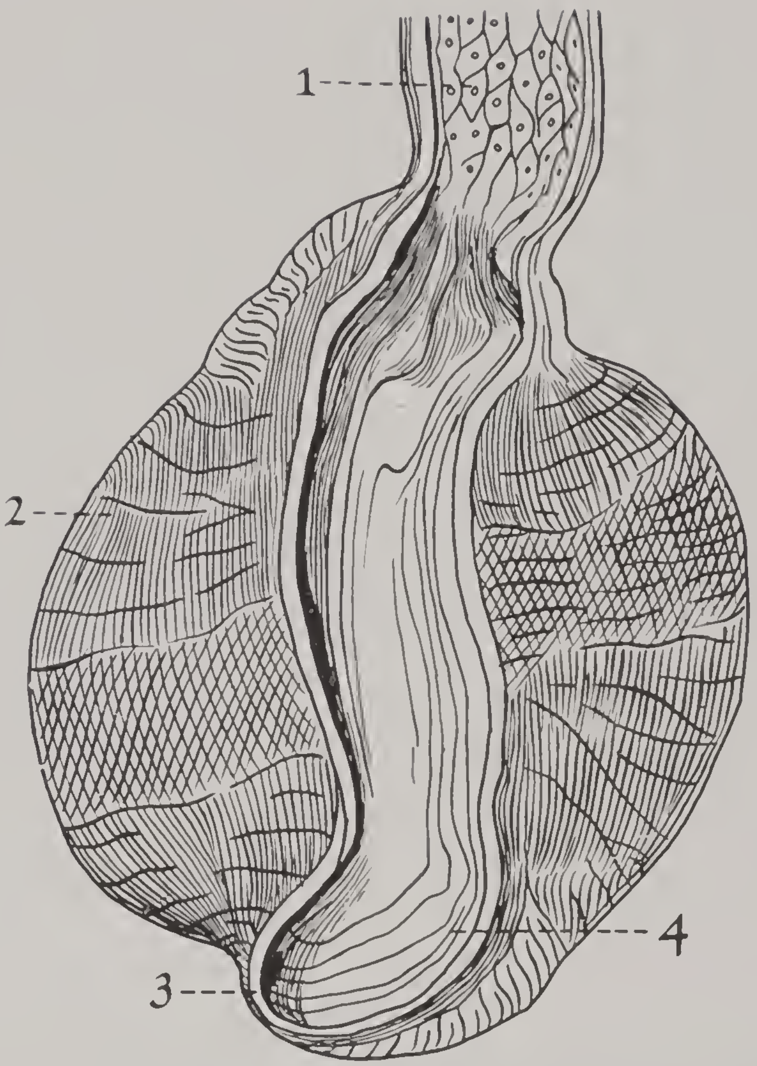

FIG. 5. Stomach of the chick. KEY: *1*, glandular stomach with papillae (each an exit of several stomach glands); *2*, muscle layers of gizzard; *3*, sinew plate uniting the gizzard muscles; *4*, lumen of gizzard. After Mangold (*101*).

The gizzard always contains grit, which is continuously taken up by the birds. It is a much debated question whether this is essential for digestion. Excitement of the birds greatly enhances the velocity of propulsion.

After the food has been collected in the crop it passes in small portions to the esophagus and thence to the proventriculus. The conditions of the emptying of the crop have been studied by Ihnen (*157*). According to X-ray observations (*156*) food is taken up by the proventriculus only when the gizzard is expanded (Fig. 6). While the gizzard is

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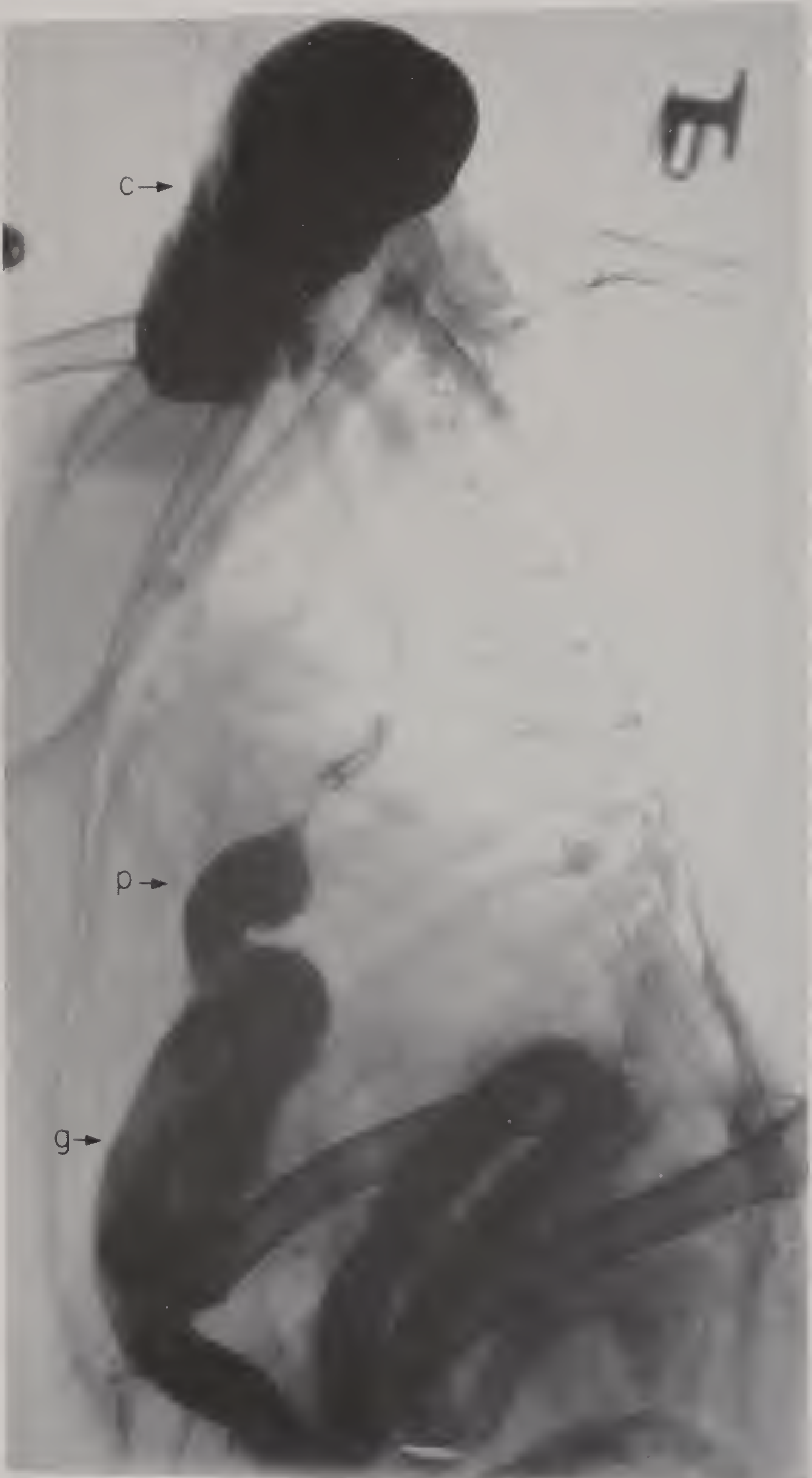
FIG. 6. Alimentary canal of the chick after feeding with barium sulfate paste. KEY: *c*, crop; *p*, proventriculus, visible by the passage of paste to the gizzard (*g*), which is in the extended state. Esophagus faintly visible. After Vonk and Postma (*156*).


c
p
g

contracted the food lingers in the esophagus in front of the proventriculus. No appreciable contents have ever been found in the proventriculus after consumption of food (*101*). It seems that the food only takes up pepsin and HCl from the walls of the proventriculus and then passes directly to the gizzard.

The relation between the pH of the contents of the gizzard and the pH optima of the pepsin of the gastric juice has already been treated. It may be mentioned, however, that there are rather large discrepancies in the values found by different investigators for the pH of the gizzard contents and in the proventriculus.

Whereas Mennega (*104*) found no pH lower than 3.68 on the surface of the fish in the gizzard of the heron and 4.18 on meat in the gizzard of the kestrel and barn owl, Farner (*158*) observed much lower values (2.0–2.60) in the gizzard of chicken, pigeon, pheasant, duck and turkey. This may depend of the kind of food taken up. Many other investigators [see Sturkie (*154*), Table 29] have observed much higher values (from 3.0 to 5.02) in the gizzard of the adult hen. The results of Hewitt and Schellkopf (*159*) confirm the latter values. However, Vonk *et al.* (*160*) found values of 2.77 in the gizzard of young chickens (up to 23 days); the pH was decreased by diets high in protein, whereas Farner (*161*) found it to increase under these conditions. In chickens older than 23 days the pH of the gizzard rises to 3.1, which is not essentially different from 3.06 in adult chicks (*161*). All these values are averages of data for a large number of animals.

Remarkable are the high pH values of 3.41–4.80 observed by Farner (*158*) in the proventriculus in view of the fact that the gastric juice is secreted here.

According to Ashcraft (*162*) the pH of the small intestine increases in feeding with dried milk products as compared to feeding with meat scraps (from approximately 6.0 to 6.5 in the duodenum and 7.0 to 7.5 in the ileum). In the cecum however, it is lowered from about 7.0 to 6.0, 5.5, or even 5.1 after consumption of 20% lactose.*

The mode of secretion in birds (pigeon and chick) has been investigated by Friedman (*163*). As in mammals, the parasympathetic nervous system (vagus) stimulates secretion. Histamine stimulates secretion of hydrochloric acid, but not of pepsin. Epinephrine (correlating its action with that of the sympathetic system) does not stimulate secretion of gastric juice. These facts are important because the conditions in this respect are different in lower vertebrates. The amount of pepsin in the gastric juice of the chick was found to be far higher than that in the pigeon. Walter (*164*) could evoke optical and acoustic, but not olfactory, conditioned reflexes for the secretion of gastric juice in the pigeon after preparation of a fistula of the proventriculus. Other investigators [cf. Sturkie (*154*)] also succeeded in obtaining gastric juice by means of fistulas.

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* Ashcraft's calculation of the average (by dividing Σ pH by the number of experiments) is not correct, the pH values being logarithms. [For correct calculation see Farner (*158*).] After correct calculation the results are more convincing than the author supposed.

Much less is known of digestion in the intestine. With the exception of lactase, the same enzymes have been found in pancreas and intestine as in mammals (cf. *154, 159*). From the walls of the small intestine of pigeon and chicken, secretin has been isolated [Koschtojanz *et al.* (*165*)]. It is unspecific: it also evokes pancreatic excretion in mammals.

The pH of the bile is slightly acid (5.88–6.10) [Sturkie (*154*), Table 9], in contrast to the pH in mammals, which is slightly alkaline. (For avian bile acids, see the contribution of Haslewood in Vol. III of this

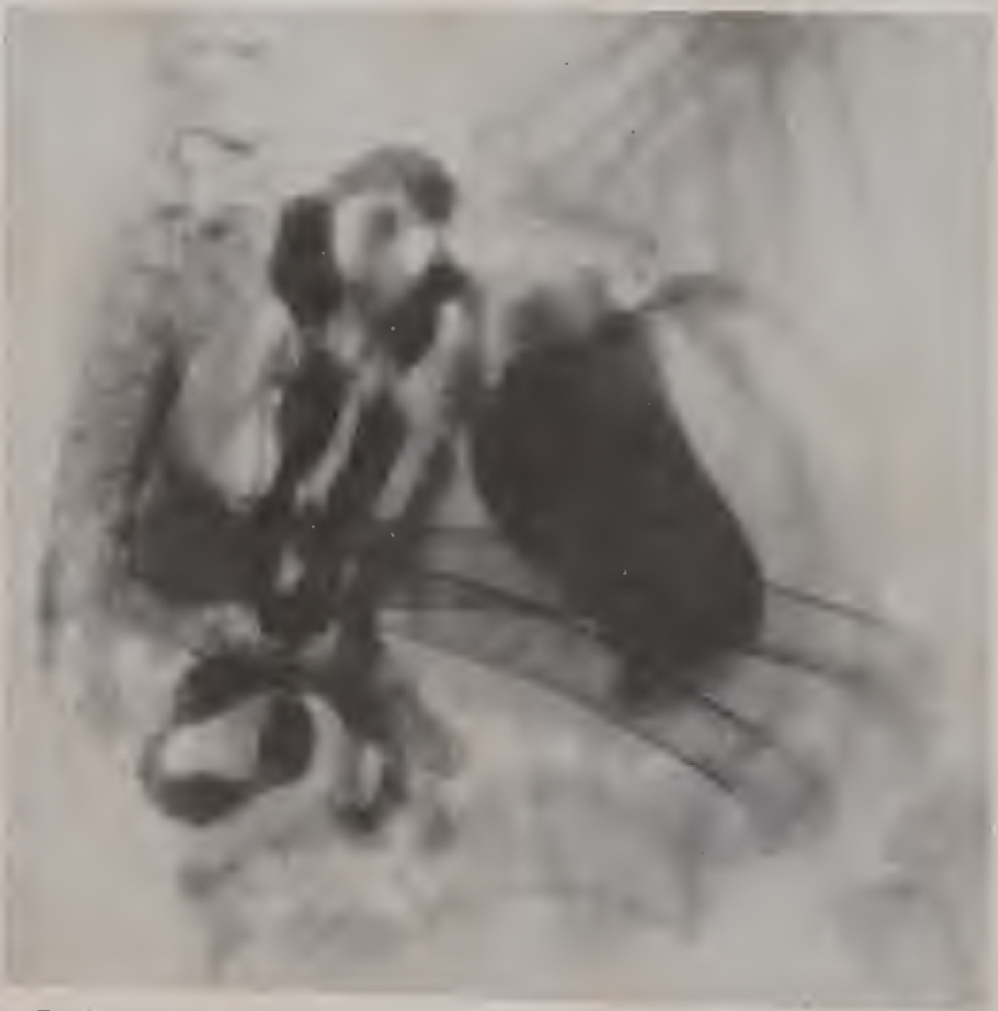

FIG. 7. Segmentations of the small intestine of the chick after feeding barium sulfate together with food. The gizzard is partly contracted. After Vonk and Postma (*156*).

treatise.) Amylase is present in avian bile; its activity in bile from the bladder is higher than in that from the liver (Farner, *166*).

If barium sulfate is mixed with the food, segmentations in the small intestine may be observed (Fig. 7). Their frequency is lower than in the cat and therefore better suited for X-ray photography. When only barium sulfate in water was given, no segmentations could be evoked (*156*). The food mass (chyme) penetrates into the ceca, which are relatively large in some forms. In the ceca, lignin and pentosan from grains can be digested by bacteria to the extent of 10–40% [Tscherniak (*167*)]. In general, digestion in the cecum seems to be less effective in birds than in mammals (here too there are considerable variations). Resistant crude fibers and true cellulose are not appreciably digested in birds. The cecum can be removed without ill effect [Dukes (*6*)], therefore its role probably is not very important. The considerably higher concentrations of B vitamins in the cecum might point to their synthesis in the cecum by means of bacteria. However, removal of the cecum did not increase the requirement for these vitamins in the food [Sunde *et al.* (*168*), Couch *et al.* (*169*)]. Water is probably absorbed in the cecum, since after its removal, the feces are less firm in consistency [Röseler, cited by Sturkie, ref. (*154*)].

It is peculiar that the African honey guides (Indicatoridae) are able by means of bacteria and yeasts in their intestinal tract to digest beeswax from honeycombs. They have no access to these combs unless the bees' nests are opened by man or by the ratel and they more or less "guide" these "foraging symbionts" to the bees' nests by means of excitement reactions (*169a*). For digestion of wax see also Section IV,E,3. That some birds produce chitinase in their stomach (*116a*) was mentioned in Section III,B.

E. Reptiles

All reptiles are carnivores with the exception of the land tortoises, some freshwater tortoises, and some lizards. Many of the carnivora among them devour their prey *in toto* without any chewing (snakes, lizards); crocodiles and some tortoises may divide their prey by biting.

This class has been much neglected in the study of digestion, probably because experimental manipulations with the animals are difficult.

For some remarks on anatomy and for a discussion of the literature up to 1940 the review of Vonk (*115*) may be consulted.

Recently Wright *et al.* (*170*) have studied the stomach secretion of *Testudo graeca* (Greek land tortoise) and of two Australian lizards (*Tiliqua nigrolutea* and *Tachysaurus rugosus*). *Testudo* is herbivorous and both lizards seem to be also, although this is not explicitly stated by the authors. In the stomach of the lizards, temporary fistulas could be made, but permanent fistulas could not be obtained.

The secretion of the stomach has a pH of about 1.0 and contains pepsin, sometimes in large quantities. However, in application of vagus stimulation and injections of parasympathomimetic drugs (pilocarpine, carbachol) and histamine, stronger stimulation and higher concentrations than in mammals are necessary to evoke secretion, and even then the effect is relatively smaller.

In the lizard *Lacerta viridis* Laur, it has been found that chitinase is secreted by the mucosa of the stomach and by the pancreas [Jeuniaux (*171*)]. These enzymes can digest the chitin of insects (meal worms) fed to the lizard. The same facts have been found for the carnivorous turtles *Emys orbicularis* and *Clemmys caspica*. Chitinase is absent in the strictly herbivorous turtle *Testudo hermanni* (*171*).

As to digestion in the intestine, Wolvekamp (*172*) has found a value of 6.5 for the pH optimum of pancreatic amylase of *Testudo*. The amylase in pancreatic extracts of the herbivore *Testudo* is two times stronger than that in the carnivore *Emys*. In absorption experiments *in vitro* it was found that monosaccharides (glucose, fructose) can pass the intestinal wall, whereas disaccharides (maltose, sucrose) do not pass. These are split slowly if in contact with the wall of the gut and then reach the serosal side as monosaccharides.

Kenyon (*173*) found a strong tryptic action in the pancreas of all the reptiles he investigated (all carnivores). A fair quantity of amylase was also present.

The small intestine of *Testudo hermanni* has been found to be an ideal object for the investigation of permeability and active transport of amino acids (*51*).

F. Amphibia

Digestion in Amphibia presents a few points in which it deviates significantly from that of the three higher vertebrate classes.

First, in many forms the glands that have the most abundant pepsin secretion occur in the esophagus. This was found by von Swiecicki (*174*) in 1876 in the frog and has since then many times been confirmed. The part played by the stomach in secretion is much smaller than that of the esophageal glands. The biological significance, i.e., advantage for the animal because of this arrangement, cannot be stated. The secretion of HCl is restricted to the stomach, so that peptic digestion can take place only there.

The greatest differences between Amphibia (and fishes) and the other vertebrate classes are concerned with the secretion of stomach juice. This question has been studied by Smirnov (*175*), Friedmann (*176*), Wolvekamp and Tinbergen (*177*), and Crombach *et al.* (*178*). All these authors fairly well agree that in Amphibia conditioned reflexes are lacking. The natural stimulus for the evocation of secretion seems to be a mechanical one: contact of the food with the wall of the stomach (*175–177*) or stretching of the stomach by the food (*178*). Regulation is local. These questions being of a physiological nature, we may refer for details to the literature mentioned above.

The relation of the pH optimum of pepsin to the pH of the stomach contents was discussed in Section III,B.

The scanty facts known on intestinal digestion in Amphibia do not show appreciable deviations from the situation in higher animals. It is only remarkable that the pancreas of the frog, a carnivore, contains a large amount of amylase quite comparable to that of herbivores (*105*). Equally remarkable is that Junold (*179*) found amylase in the saliva of the frog, which was collected on silkpaper brought into the mouth. The significance of this fact is not clear. The enzyme could (the frog being a carnivore) act only on glycogen and this can be set free only after the proteins have been digested.

G. Fishes

The points in which the digestion of fishes deviates from that of the Tetrapoda are: the regulation of the gastric secretion, the structure of the pancreas, the presence of appendices pyloricae in many forms, and the absence of the stomach in several families and species.

1. *Secretion*

In hunger continuously a small amount of gastric juice is secreted, but hungry dogfishes which have pursued their prey without capturing it have no more gastric juice than control animals which have not chased; thus conditioned reflexes (psychic secretion) would seem to be lacking (*180*). The same has been found for skates (*181*). [In the stomachless *Fundulus* pilocarpine is a stimulus for pancreatic secretion (*182*)]. Thus, as in amphibians, the conditions for secretion of stomach juice are quite different from these in mammals and Sauropsida (for details, see references *180–182*).

2. *Pancreas*

In Elasmobranchii the pancreas is a compact organ as in Tetrapoda. However in Teleostei, it is divided into ramified threads, often very thin, which mostly follow the blood vessels and may even penetrate into the liver. In some species, like the herring, the rami are so thin that they can be demonstrated only in microscope preparations (pancreas disseminatus) so that the organ was long thought to be absent. The state of ramification and the dimensions of the organ are very different in various species and moreover very variable in one species (*190*).

3. *Appendices pyloricae*

In most of the teleosts, near the border of stomach and duodenum the duodenum carries a number of appendixes (appendices pyloricae, Fig. 8) which may vary in number from three (in the perch) to many hundreds (in the Gadidae). They open into the duodenum and are traversed by many folds originating from their walls. As for a long time the pancreas of fishes was unknown, Cuvier proposed the theory that the appendixes were a compensation for the lack of a pancreas. The pancreas has now been found universally in fishes, the structure of the wall of the appendixes is equal to that of the gut (*115, 190*), and food penetrates into it (*183*); therefore it can hardly be doubted that the appendixes

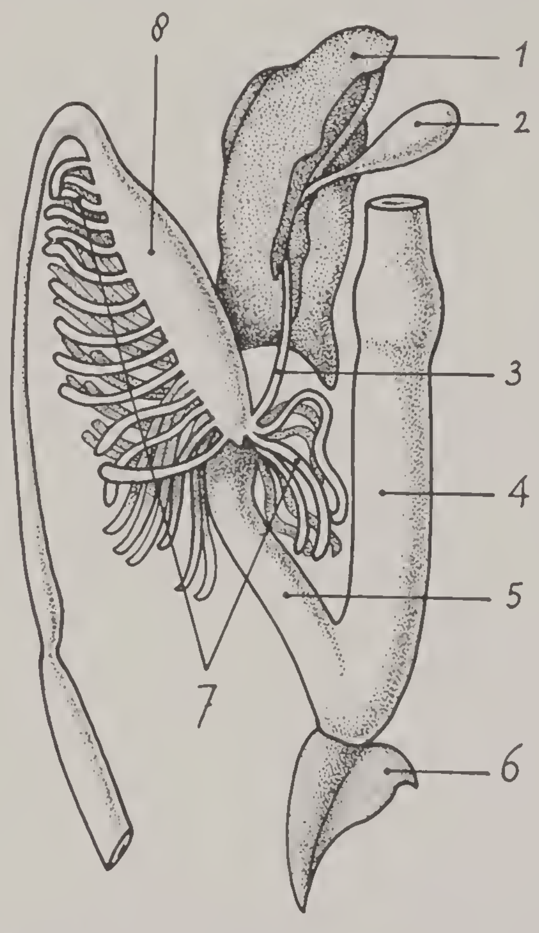

FIG. 8. Proximal digestive tract of *Trutta lacustris* (trout). KEY: *1*, liver; *2*, bile bladder; *3*, bile duct; *4*, proximal, and *5*, pyloric, parts of stomach; *6*, spleen; *7*, appendices pyloricae; *8*, proximal midgut. Pancreas omitted. After Bütschli, drawn from Vonk (*115*).

function by enlarging the surface of the intestinal mucosa and that the enzymes found in them (chiefly endo- and exopeptidases) are of pancreatic origin. Still it is difficult to prove the latter view because of the small size of the appendixes and the penetration of the pancreas between them. Some indications supporting this view have nevertheless been found (*184*) in the perch.

4. *Stomach*

The stomach is lacking in Holocephali and Dipnoi, in the Teleostei in the families of Cyprinidae (carps) and Cyprinodontidae, and also in a number of genera scattered among other families [reviewed by Hirsch (*185*)]. In such animals appendices pyloricae are absent and the bile duct opens into the small intestine directly behind the esophagus. However, in these fishes the first part of the small intestine is often much wider than the following parts and may simulate the presence of a stomach. In fact this wider part has sometimes been believed to be a stomach by biochemists unfamiliar with the anatomy of fishes (*186*). But histological examination shows the identity of the wall with that of the following narrower part of the gut [Al-Hussaini (*187*)]. Nor have pepsin and HCl ever been found in this part of the gut (*182, 188, 189*), but research on this point has not been extensive.

Barrington [(*190*), p. 132] considers this absence of the stomach to be not a primitive condition, as was suggested earlier, but a secondary one—a reduction. What has caused this loss is uncertain and it can hardly be considered an advantage.

In other points there is much similarity between conditions in fishes and in higher vertebrates (*105, 115*). The relations between pH and digestion in the stomach were discussed in Section III,B. Pepsin of salmon and of tuna has been recently obtained in crystalline form (*191, 192*) according to the procedures of Northrop *et al.* (*88*). Fish pepsin differs from bovine pepsin in crystal formation, in composition, and (slightly) in specificity. The optima are the same as for bovine pepsin.

5. *Course of Digestion*

Schlottke (*183*) has drawn attention to the significant role of food enzymes in aiding intestinal digestion in fishes. Margolis (*193*) has shown that an extensive collaboration of bacteria in the digestion of fishes is improbable: in fishes investigated by him bacteria were present in the gut only after feeding but not in hunger (*Ameirus, Salvelinus*). On the other hand, presence of lichenase (*188*), salicinase, and alginase (cited in reference *190*, p. 142) is reported for herbivorous fishes. With regard to Margolis' statement, it must be borne in mind that in ruminants the condition of the intestinal flora is very dependent on the kind of food, so that absence of bacteria in hunger may not exclude some collaboration of bacteria after feeding.

With regard to the course of proteolytic digestion in fishes, the older but admirable work of Van Slyke and White (*194*) still best elucidates this process. Dogfishes were fed known quantities of meat and killed after 6, 12, 24, 28, and 72 hours. The end of the digestion was reached in 2–3 days [this agrees with results on pikes (*105*)]. At these different times and at successive places in the gut, the length of the peptide chains (after separation from the proteins) was determined. After 6 hours the peptides in the stomach were pentapeptides (average). The intestinal contents were scanty and as 25% of the fed proteins had disappeared, the authors concluded that these had been adsorbed in the stomach. It is possible that proteins could have been transported to the small intestine and rapidly digested and absorbed there. On the other hand absorption in the fish stomach is not improbable; it has been found for fat in the dogfish and the plaice (*195, 196*). After 12 hours much digested and undigested protein had reached the intestine; peptides in the stomach were now tripeptides, in the gut somewhat shorter. After 24 hours di- and tripeptides were in the stomach; in the later stages peptides smaller than these are found neither in stomach nor intestine. This points to final cleavage in the intestinal wall, which has also been claimed for mammals. Compared with digestion in the dog, digestion in the dogfish is 8 times slower (*194*), but if the temperature difference of 20° is taken into account (which may cause a difference of 4–9 times in reaction velocity) the efficiency is about equal in dog and dogfish.

A remarkable fact is that some fishes (e.g., salmon, *Petromyzon*) which cover distances up to 1100 km. in migrating to their spawning places, do not feed during migration. Their digestive tract gradually disappears and, with other reserves, is used as fuel for the strong muscular movements. Individuals of such species often die after spawning (*197, 198*).

Digestion in fishes has been investigated far more extensively than in reptiles and amphibians. For an elaborate recent review Barrington (*190*) may be consulted.

IV. Digestion in Invertebrates

The diversity in anatomical structure and physiological organization in invertebrates is so great that only some of the most important features can be treated—features in which the digestion in invertebrates differs from that in vertebrates. [For general information cf. references (*12, 13, 199–202, 204–207*); for Protozoa (*208, 208a*)*; for Crustacea (*209, 210*); for Insecta (*79, 211–213c*). Recently an exhaustive treatment of digestion in the animal phyla has been given by von Buddenbrock (*207*). Valuable data on the physiological anatomy may be found in some older works (*214–216*).]

Absorption in invertebrates has been treated in Section II,B,2. A treatment of the extremely divergent feeding mechanisms is beyond the scope of this book [cf. references *199, 201, 214, 215;* for crustaceans (*209*) and for ciliary feeders (*203*)]. An exception has been made for the phenomenon of phagocytosis, which is of great general importance.

A. Phagocytosis and Intracellular Digestion

The smallest animal organisms which may be considered to consist of one cell (Protozoa), have no special digestive organs, although in some a cell mouth and a kind of pharynx as an entrance to the cell are present. Neither have they very special means to capture their prey. Foraminifera may do so by a net of fine pseudopodia. Simple protozoa like *Amoeba* send out pseudopodia with which they engulf food particles. Leukocytes in the blood take up bacteria in the same way; Metchnikoff called this phenomenon phagocytosis, and the term has since been applied to the mode of feeding of unicellular and simple polycellular animals, in which it persists.

In Protozoa the ingested food is mostly taken up in a vacuole into which enzymes are secreted (Fig. 9). The products of digestion permeate through the wall of the vacuole into the cell. Often the captured prey is killed during an acid phase of the vacuole and digestion takes place when the reaction of the vacuole has become slightly alkaline [Nirenstein (*208*)]. Phagocytosis by Protozoa can be easily observed microscopically *in vivo*. Besides food, phagocytosing cells may also take up particles of a suspension of India ink (carbon), carmine, gold etc. As a result of studies of ingestion from such suspensions, it can be stated that the cells of the gut of lower invertebrates still possess the faculty of taking up solid particles and the conclusion is drawn that they act in the same way with small food particles. This can be confirmed by observing the uptake of fat globules (which can be stained), chloroplasts, etc.

More precisely, we speak of phagocytosis if particles taken up are larger than the colloidal size (diameter > 100 mμ). The taking up of particles of colloidal size (diameter 1–100 mμ) in a way that can be made visible microscopically is nowadays called pinocytosis. Below a diameter of 1 mμ, we speak of absorption [(*201*), p. 25]. In experiments

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* See also Burck (*217*).

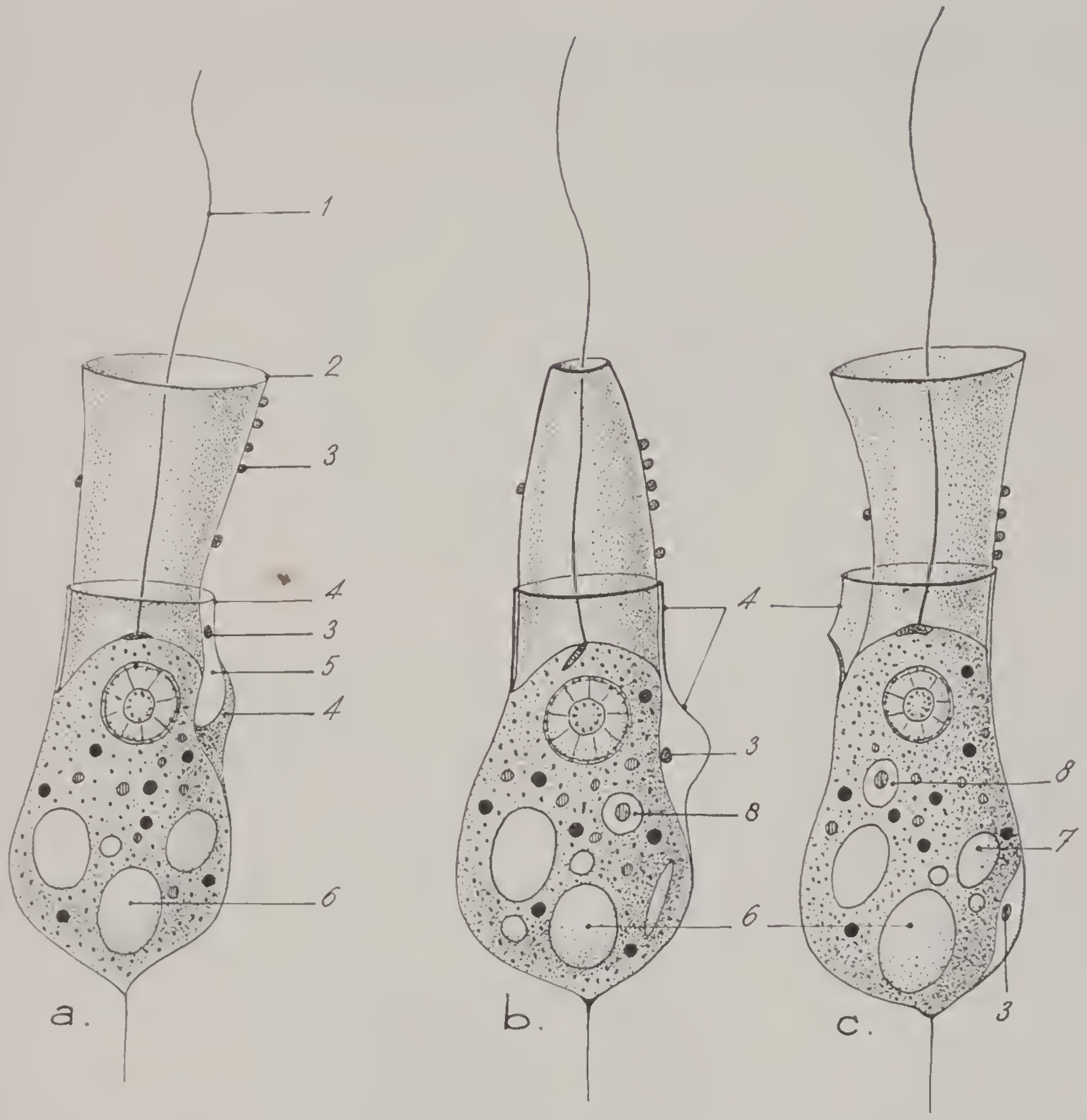

FIG. 9. Stages (*a–c*) of ingestion of food by *Codonosiga botrytis* (Ehrbg.) (Choanoflagellatae). KEY: *1*, flagellum; *2*, collar of the cell. The food particle (*3*) glides along the outside of the collar, is taken up in the space (*5*) between the mucous layer (*4*), and the cell wall, and is transported with this space to the base of the cell and taken up there; *8*, in *b* and *c*, food particles which have already been taken up by the cell; *6*, noncontractile vacuole; *7*, contractile vacuole. After Burck (*217*).

on phagocytosis care must be taken to control the size of the particles: no colloidal particles should be available for uptake which, after being lumped together in the cell to microscopic dimensions, might seem to have been taken up as a result of phagocytosis. Especially objectionable is the use of carmine as this is somewhat soluble at the slightly alkaline pH of sea water and of some kinds of fresh water.

Unless the prey is very small, preliminary digestion in the gut is necessary. The gut of such phagocytosing animals is narrow, so that good contact of the predigested food with the phagocytosing cells is afforded. Such feeding by phagocytosis [cf. reviews in (*199–201*)] is found in sponges (*218–220*) (Fig. 10), Coelenterata [corals, sea anemones (*221, 222*), jellyfishes], Plathelminthes (*223*) (a class of lower worms, see Fig. 11), and Lamellibranchiata [mussels (*67, 224, 224a*)]. In gastropods

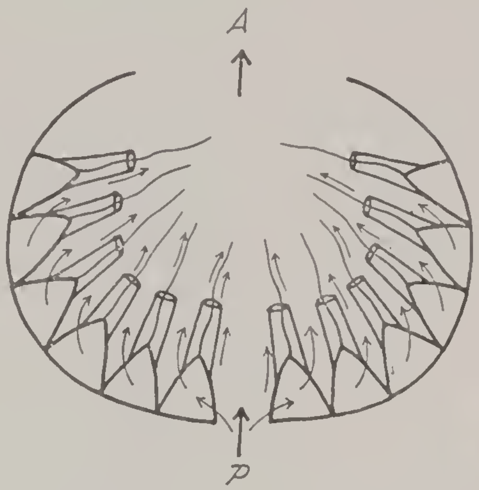

FIG. 10. Chamber of a sponge, lined by cells (choanocytes), similar to the single cells of Choanoflagellatae, which take up their food in the same way. The movements of the flagella produce a water current which enters the chamber at *P* (prosopyle) and leaves it at *A* (apopyle). This water current provides food particles and oxygen. After Yonge (*199*).

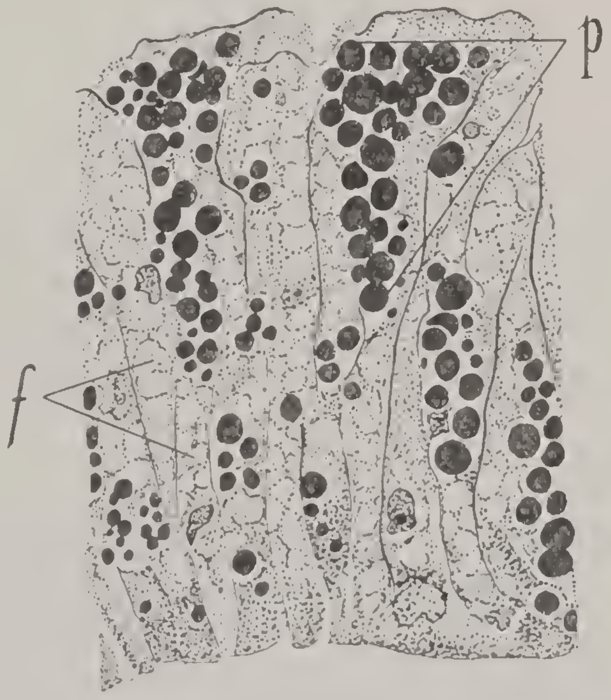

FIG. 11. Phagocytosis of protein particles by intestinal cells of a triclad flatworm (*Planaria dorotocephala*). KEY: *p*, protein particles; *f*, vacuoles which contained fat globules (removed by the fat solvents used). After Willier *et al.* (*223*).

(snails) feeding by phagocytosis exists in some forms and is absent in others (*225, 226*). The question whether it is present in *Helix* is very controversial; it is the conclusion of this reviewer that under favorable circumstances phagocytosis can play a role in digestion in *Helix*, but under such difficulties that it may have no great value in the digestive process of the animal. It has been reported for echinoderms, but is decidedly lacking in Cephalopoda, Crustacea, and Insecta. However, digestion by phagocytosis is still found in *Amphioxus;* here it was demonstrated by the taking up of spermatozoa, the nuclei of which can be detected by the Feulgen reaction on nucleic acids (*227, 228*). Even the intestinal cells of young mice probably have this faculty [Von Möllendorff (*229*)]. Not very much is known of the process of intracellular digestion which must follow phagocytosis. Some facts and figures may be found in references *219, 220,* and *220a* (cf. also reference *204*, pp. 489 and 490).

B. Invertebrates with a Well-Developed Intestine

The chief difference between these animals and vertebrates is that in the vertebrates the various chemical agents are secreted seriatim (by salivary glands, stomach, pancreas, intestine, bile from the liver) and develop their action in different parts of the intestine, whereas in invertebrates secretion is far less localized: the enzymes mostly work together in one place and the intestinal juice is a mixture of many different ferments (*201, 202*).

Neverthcless there are invertebrate groups in which some differentiation has come about. The most important of these differentiations is the midgut gland or hepatopancreas, also called digestive diverticula. This gland occurs in all the classes of the Mollusca, in Crustacea, Scorpionida, and Arachnoidea. It is lacking in higher worms (Annelida), echinoderms, and insects. It is a glandular organ which contains alveoli of active cells separated from each other by a rather large mass of connective tissue. The alveoli are connected to the gut by a system of ducts, which may conduct secretions from gland to gut and also small food particles from the gut to the interior of the gland. These propulsions of secretions and of food particles are mostly performed by ciliary movement. The epithelium of the alveoli has a versatile function: it serves for the formation and secretion of enzymes and also for absorption (e.g., in Crustacea, Gastropoda, and Cephalopoda). In Lamellibranchiata and some of the Gastropoda the epithelium is capable even of phagocytosis. The functions may be performed by different kinds of cells or by one undifferentiated type of cell (*199, 201, 224a*).

The gland also serves for storage, mostly of glycogen, fat, and calcium [Renaud (*230*)]. Moreover it has important metabolic functions. Synthesis of fatty acids and cholesterol, transaminations, degradation of nucleic acids, formation of urea and uric acid, and oxidation of various metabolites have been found (summarized in references *210*, *230*). Therefore the name digestive diverticula should be abandoned; as the organ is comparable in some respects to the vertebrate liver and in others to the vertebrate pancreas, the name hepatopancreas seems the most appropriate. Still it must be noted that the gland has also the function of absorption, which neither the liver nor the pancreas of vertebrates exhibit.

The presence of salivary glands in many invertebrates is another evidence of a degree of differentiation. If enzymes are secreted, these are mostly amylases, but proteinases occur also. Salivary glands may also secrete acid (cf. Section IV,C), poison for killing or benumbing the prey, as in Cephalopoda [Romijn (*231*); for recent work on these glands in Cephalopoda, cf. references 60–66 cited in reference *204*]. In cephalopods also some localization in the secretion of proteolytic enzymes is found. The hepatopancreas contains proteinase and polypeptidases; the pancreas (a gland following on the hepatopancreas) has only peptidases and inactive proteinase, and the enterokinase necessary for its activation is localized in the wall of the cecum (*231*).

A peculiarity of the midgut and hindgut of many invertebrates (chiefly insects) is that they secrete the thin peritrophic membrane which surrounds the intestinal contents and the feces (*79*, *204*, *207*, *210*, *211*). Electron microscopic studies have revealed that the membrane consists of a network of fibers in which an amorphous material is embedded. The presence of chitin has been demonstrated by the chitosan test (*231b*). It seems not impossible that the fibrillar component consists of chitin and the amorphous ground substance of protein (*231a*). Recently the presence of chitin has been confirmed by means of digestion with bacterial chitinase (*231c*). In this way 4–13% chitin was found in the peritrophic membrane of insects, whereas the protein content (calculated from nitrogen determinations) was between 21 and 47%. Moreover, about 8% of ash was found (in *Bombyx*) and the presence of a mucin seemed probable (*231c*). The function of the membrane is probably to protect the intestinal wall against contact with rough particles.

C. DIFFERENCES BETWEEN CHEMICAL DIGESTIVE AGENTS OF VERTEBRATES AND INVERTEBRATES

In general the same enzyme systems for proteolysis are present in vertebrates and invertebrates (*202*, *232*). However, pepsin has nowhere been found in invertebrates. Both vertebrates and invertebrates possess nearly exclusively α-amylase which (in contrast to plant amylases) needs activation by salt (mainly NaCl, other salts are less effective) (*202, 233*). It is peculiar that animal amylases hardly attack intact starch grains; even grains injured by chewing are not much better digested (*234, 235*). Probably *in vivo* the enzymes of the food (among them β-amylase) are an effective help in the natural process. It has long been recognized that invertebrates and especially insects have far more possibilities for the digestion of di- and trisaccharides with β-linkages than vertebrates (*204*). Moreover, in general the optima of their enzymes coincide with the pH of the intestinal contents. This picture is confirmed by many modern investigations cited in the above-mentioned reviews on insect digestion (*213, 213c*). As recent proofs for this confirmation two papers may be cited: that of Ehrhardt and Voss (*235a*) for the first point; that of Krishna and Saxena (*235b*) for the second. The former authors have investigated qualitatively the carbohydrate digestion of two roaches (Blattoidea) by means of paper-chromatography and found several α- and β-dissaccharases present. Krishna and Saxena investigated the enzymes of *Tribolium castaneum* (a floor beetle) and found that the pH of the intestinal contents (pH 5.2–6.0) coincided fairly well with the optima of the various enzymes. In general in invertebrates the pH of the intestinal contents is slightly acid (*206*). In the minority of the cases these pH values are slightly alkaline. Thus Van Weel (*235c*) found in the crab *Thalamites* optima for lipase, carbohydrases, and proteinases between pH 7 and 8.5. Unfortunately the pH of the intestinal contents has not been determined. The lipases are very similar in both groups; in invertebrates they have somewhat more the character of esterases (*202*).

In accordance with the absence of pepsin, no free HCl is secreted in invertebrates. Some carnivorous snails (*Dolium, Tritonium,* and many others) secrete sulfuric acid in concentrations up to 1 *N*, or aspartic acid from their salivary glands. This acid, however, does not serve for digestion but as an aid in dissolving the shells and other calcareous structures of the prey of these animals [cf. discussion by Jordan (*214*), p. 285].

In Crustacea (Decapoda) emulsifiers have been found (*236*) which have been supposed to be bile acids (cf. reference *210*, p. 297) because of their physicochemical properties, the liberation of taurine in hydrolysis, and their chromatographic behavior. The identity of these emulsifiers in *Cancer pagurus* is being studied at present (*236a*); the preliminary result is that there are at least two of these compounds. Both contain taurine; one contains a C_{10} saturated fatty acid and the other an unsaturated C_{12} fatty acid. There are several indications that both must contain a third still unknown component (*236a*). The nature of the linkage with taurine is not yet clear. Unidentified emulsifiers have also been found in other groups of invertebrates (*65, 236*). These emulsifiers (cf. also *38a*) may play in invertebrates the same part as the bile in vertebrates, i.e., aid in digestion and absorption of fat and fatty acids (*210*). Probably they originate in the hepatopancreas, if present.

A remarkable structure is the so-called crystalline style in some mollusks, especially Lamellibranchiata. This is a rod of protein gel which contains a strong amylase (*199–202*); it was found later that it may contain also lipase (*237*) and cellulase (*238, 239*). The style is secreted in a style sac (or in a fold of the gut); it is resistant at the low pH in the sac but dissolves at the higher pH of the intestine, into which it is slowly moved by rotation effected by cilia. During this rotation the free end of the style rubs against the "gastric shield," a chitinous plate covering a part of the stomach wall. The food adheres to the upper part of the style and is digested there (*200*).

For a long time it has been thought that in the Lamellibranchiata the crystalline style is the sole source of digestive enzymes, that these are exclusively carbohydrases, and that the digestion of proteins is intracellular. However, Mansour (*239a*), Mansour-Bek (*239b*), and Zaki (*239c*) found proteinase, peptidase, and an esterase in the stomach juice of species of *Tridacna* and *Unio,* which, according to these authors, originate from the hepatopancreas (probably by means of holocrine secretion). The potency of these enzymes is much greater than that of an extract of concentrated amebocytes, so that these cannot be the origin of the enzymes found in the stomach. Thus it seems, that an extracellular predigestion exists at least in part of the lamellibranchs. [Yonge (*239d*) has disputed this view.] The conclusion [Mansour (*239a*)] that the whole idea of an intracellular digestion in Lamellibranchiata must be abolished is unwarranted, however, because several authors (cf. *199–201*) have beyond doubt established phagocytosis in lamellibranchs. The digestion in mollusks has been shortly reviewed by Van Weel (*239e*). Shortly an extracellular proteinase has also been found in *Helix pomatia* (*239f*).

According to Berkeley (*239g*) the style, as a result of acid hydrolysis. yields in addition to protein: glucuronic acid, sulfate, and a hexosamine; in view of this analysis and of its solubility properties, it is probably a mucin. The function of the organ may be to prevent waste of enzymes (the lamellibranch stomach is in open connection with the surrounding water) and to mix and triturate the food (*203, 224a*). In some animals the style contains spirochetes which may be partly responsible for its enzyme content (*203*).

D. SECRETION OF ENZYMES

Concerning this physiological problem it may suffice to mention some recent papers from which the older literature (cf. *240*) may be found easily.

Van Weel (*241*) has pointed out a rhythmic merocrine secretion in the hepatopancreas of the crustacean *Atya*. Less distinct is the rhythm in *Achatina* (*242*). This author also studied the effect of diet on the production of enzymes in *Achatina* (*243*). In adult animals on a diet of starch the quantity of amylase is increased; curiously, on a protein diet the amount of proteinase is decreased (*243*). In young animals, which feed on plants, the ratio amylase:proteinase is greater than in adults, which are omnivores (*244*). Whether the change in this ratio is caused by, or is the cause of, the change in diet is unresolved. Some critical comment on the problem of rhythmic secretion can be found in references *210* and *235c*.

Some remarks on secretion in insects are given by Waterhouse (*213*); for secretion in *Tenebrio* and *Dytiscus*, see Dadd (*244a*).

E. DIGESTION OF SPECIAL KINDS OF FOOD

1. *Cellulose, Hemicellulose, Chitin, Wood*

Vertebrates (with a few exceptions, cf. Section III,E) are unable to produce cellulase and chitinase. Insofar as cellulose and hemicellulose are digested by vertebrates, the digestion takes place by the action of bacteria. The presence of a cellulase in the intestine of an invertebrate (*Helix pomatia* L, the snail) was discovered in 1898 by Biedermann and Moritz (*245*). This fact has been confirmed by many observers for different invertebrates. In 1923 Karrer undertook an extensive study of the action of the intestinal juice of *Helix* on lichenin, a hemicellulose prepared from the cell walls of *Cetraria islandica,* and on genuine cellulose [full literature and short review in Vonk (*202*)]. Cellulose (filter paper) is attacked with difficulty, hemicellulose rapidly. The pH optimum is 5.28 which nearly coincides with the pH of the intestinal contents. Karrer and Hofman (*246*) also discovered chitinase in the intestinal juice of *Helix* (cf. *202*).

More recently Florkin and his colleagues (*246a, 247*) have found that the cellulolytic action of *Helix* intestinal juice is caused by the action of bacteria. The bacteria were isolated and pure cultures were made, into which the cellulase was secreted. The same facts were stated by Jeuniaux for the chitinases of *Helix* and of an isopod which were believed until then to be animal secretions (*248, 249*). It seemed therefore that the concept of invertebrate cellulase and chitinase was severely impaired. However, some animals remained in which cellulase was found although absence of bacteria could be definitely established [*Teredo, Limnoria lignorum*, a wood-boring isopod (*250, 251*)]. Perhaps the most convincing of these cases is that of *Ctenolepisma lineata*, a tropical silverfish (insect) investigated by Lasker and Giese (*252*). They found in the nymphs of this animal a digestive capacity for cellulose of approximately 80% (which equals the value in ruminants). No cellulolytic bacteria could be found in the intestine and aseptically reared nymphs took up C^{14} from C^{14}-labeled cellulose. It seems therefore that, despite the case of *Helix*, the original notion that certain invertebrates are able to secrete a cellulase in their digestive tract, may be revived. The same applies perhaps to chitinase. In fact *Maya squinado* and *Cancer pagurus* are able to produce chitinase in the epiderm, where it can hardly be ascribed to bacteria (*253*). Extracts of hepatopancreas of *Eriocheir* (*254*) and of *Maya squinado* (*255*) also contain chitinase and chitobiase (bacterial or glandular origin still uncertain). Moreover in *Lumbricus terrestris* it could be shown that a profound modification of the bacterial flora by antibiotics has no influence on the chitinase content of the intestinal juice and of the wall of the gut. Thus the animal origin of chitinase in *Lumbricus* has been well established by these experiments, although in the normal animal also chitinolytic bacteria are present (*256*). That also insects are capable of producing chitinase is proved by its presence in the molting fluid between the old and new cuticles (*257*). The same has been found for the silkworm (*Bombyx mori*) (*258*). The pH optimum is 5.4. Myers and Northcote (*259*) have recently reported on the action of *Helix* juice on cellulose, chitin, and 14 other carbohydrates, apparently without knowing of the work of Florkin and colleagues [cf. also Holden *et al.* (*260*)].

The question of secretion of cellulase in snails has been investigated anew by Parnas (*260a*) for *Levantina hierosolyma*. He found that washing of the digestive tract prior to homogenization resulted in the complete cessation of cellulolytic activity in the salivary gland and the crop-stomach, but not in the hepatopancreas. In snails treated with antibiotics the experiments had the same result. Cellulase activity was found in culture media in which portions of the crop-stomach and hepatopancreas had been placed. If the snails had been treated with antibiotics, cellulase was found under the same circumstances only in tubes containing hepatopancreas. Thus it may be concluded that the cellulase in snails is of double origin: partly from bacteria and partly produced by the animal's hepatopancreas. In view of the evidence given above, the general conclusion may be drawn that several invertebrates (probably including the original case of *Helix*) are able to produce cellulase but that its action in many cases may be reinforced by presence of cellulolytic bacteria.

Several Protozoa are able to digest cellulose, which they phagocytose in the form of algae or of decomposition products of plants. However, as especially the latter material is often contaminated with cellulolytic bacteria, it is hard to make out whether the cellulase in the protozoan vacuoles is of animal origin or is provided by the action of bacteria (*261*, *262*). Cf. also the important work of Mast (*262a*) on *Paramecium*.

Cellulase is important also in animals which digest wood, such as shipworms [*Bankia* (*238*, *239*); *Teredo* (*250*)] and the isopod *Limnoria* (*251*), which may cause severe damage to wooden ships, breakwaters, and piers.

Many insects also are able to live in wood and therefore must be able to attack it and to derive nutrients from this substrate. Lignin seems never to be attacked by insects [(*211*), p. 278]. As to cellulase two possibilities are realized: some wood-living insects secrete a cellulase (*263*), others (e.g., termites) are dependent on the action of bacteria. A third group comprises such animals as *Cossus* which can attack the wood mechanically but use as food only soluble carbohydrates that occur in the wood (*263*). If these soluble products have been previously extracted with water of 60°, *Lyctus* larvae fail to grow; if starch is also extracted, they die (*264*). Accordingly, the cerambycid larva *Macrotoma palmata*, which contains an active cellulase, can live in the heartwood of trees, where the sugar concentration is only 0.5 to 0.7%, whereas *Xystrocera globosa*, which has no cellulase, is able to live only in the sapwood of the same trees, which has a concentration of 10% soluble carbohydrate [(*211*), p. 279].

2. *Keratin, Collagen, Silk*

The clothes moth lives on wool, i.e., keratin, which is resistant to the attack of proteinases. Extracts of the intestine of this moth have no action on keratin. That nevertheless wool must be digested by the animal is clear from its mode of living and from the fact that the feces contain twice as much sulfur as the wool from which they must have been derived (Titschakek, cited in reference *211*). The explanation is that in the intestine of the moth (*Tineola bisselliella*) a strongly negative redox potential has been found which enables splitting of the —S—S— bridges, which connect the chains of keratin, by transfer into —SH (*265*). The proteinase of the clothes moth larvae (in contrast to trypsin) is not inhibited by —SH groups. After artificial reduction, keratins are also split by the tryptic enzymes of vertebrates (*265*), a result which confirms the findings in *Tineola.* These results have been confirmed in recent investigations (reviewed in reference *213*). The negative redox potential is of the order of —109 to —230 mv. at pH 7.0. *Mallophaga,* which infests birds, digests feather keratin in a similar way (*213, 266*). Cf. addendum (Powning *et al.*).

Collagen is the main constituent of fibrous tissues. It is resistant to vertebrate trypsin but is slowly attacked by pepsin. In blowfly larvae (*Lucilia*) a collagenase is present distinct from the tryptic enzyme. As it is also present in the feces of bacteria-free larvae, it must be secreted by the animal (*267, 268*). It must be helpful in the extraintestinal digestion of these larvae. These facts have been confirmed recently, and collagenase has also been reported from other fly larvae (cf. *213*). Although it is possible to rear blowfly larvae aseptically on a semisynthetic diet, in normal circumstances bacteria that develop on the infested meat must aid in its digestion (*269*).

The larvae of various clothes moths and of *Anthremus muscorum* (a dermestid beetle) are able to digest silk, a simple but resistant fiber protein. The enzymes are unidentified (*270*).

3. *Wax*

Wax is a mixture of hydrocarbons (C_{35}–C_{31}), free wax acids (C_{24}–C_{32}), and esters. The esters are composed of C_{16} and C_{24} acids and C_{24}–C_{34} alcohols [Warth (*271*)]. It is resistant to lipase. Nevertheless *Galleria* (wax moth) larvae are able to live on the wax of honeycomb. This contains 60% wax, whereas the excreta of the wax moth contain only 28% [(*211*), p. 180], a finding which proves digestion. Bacteria probably play a part in attacking the wax (*272, 273*); this was verified by Florkin *et al.* (*274*). It is very remarkable that a small group of birds (chiefly African) live on a diet consisting partly of beeswax. These birds "digest" the wax by means of bacteria and yeasts in their intestinal tract (cf. Section III,D and reference *169a*).

F. Extraintestinal Digestion

Several invertebrates have the faculty of digesting their prey outside the body by ejecting enzymes on it or injecting enzymes into it. These enzymes may arise from the saliva or from the intestine and are mostly of a proteolytic nature. The action may or may not be supported by movements of the mouthparts for distributing the enzymes or for chewing. (Often before the digestion a poison may be injected for killing the prey.) Many examples are to be found in insects: blowfly larvae (*267, 269*) (ejection); larvae and imagoes of *Dytiscus* (*275, 276*), *Notonecta,* and *Carabus* (injection). If a larva of *Dytiscus* feeds on a transparent insect, the repeated movements of the brown digestive fluid to and from the prey may be observed (*275*). [More examples are to be found in references *79, 201, 211.*] Of noninsects, *Octopus* (ejection) some Turbellaria, and spiders (injection) may be mentioned (*201, 231*). If injection takes place, often the mouth of the animal is shut and the secreted and ingested fluids pass through canals in the mouthparts.

Larvae of *Miastor* and *Cecidomya* form conglomerates and eject together onto the surrounding wood or stems which are supposed to be attacked by this juice (*277, 278*). They have been taken as examples of animals which eject carbohydrases, but bacterial action has not been excluded.

Among vertebrates no animals with extraintestinal digestion are known.

G. Reduction of Alimentary Canal and Enzymes

If animals (like intestinal parasites) live in surroundings where food of high quality is amply present, there may result a reduction or total absence of their alimentary canal. They then take up the surrounding food through their body wall. Such animals may be called parenterals (from *parum* = few) [(*201*), pp. 59, 60]. So the whole group of Cestoda (tapeworms) is without gut; in other groups (Nematoda, Copepoda) only some forms are deprived of an intestine.

In blood parasites like *Trypanosoma evansi* (Protozoa, Flagellata) there is a loss of enzymes (amylase, lipase, disaccharases, proteases, partly) which are no longer necessary for digestion (*279*).

H. Symbiosis

We have seen that in ruminants and in many invertebrates bacteria collaborate in the digestion of various materials (chiefly cellulose, but also wax and in some cases protein). In these cases the bacteria find in the intestine of the host a favorable environment whereas the host profits by their metabolites, so that both host and bacteria have advantages from the relationship. This coexistence with mutual advantage is called symbiosis, a term first applied by de Bary (1879). Of course many gradations are possible between symbiosis and parasitism. In ruminants the advantages for the bacteria and protozoa are limited because ultimately they are digested by the host. Sometimes the host produces substances which may restrict the number of bacterial species.

Complicated forms of symbiosis are found if insects make use of "fungus gardens" for the digestion of vegetable matter. Such cases of symbiosis are reviewed by Buchner (*280, 281*). If endosymbionts (microorganisms within the cells of the host) are present, they may aid also in the synthesis of necessary metabolites (e.g., sea anemones, sponges, roaches). But free-living symbionts also may do so (synthesis of protein and B vitamins in the intestine of ruminants and of vitamin K in all mammalian intestines).

V. Carnivorous Plants

These plants, belonging to a limited number of families, are able by means of various contrivances to capture insects and other small animals and to secrete enzymes by which the prey is digested; digestion is followed by absorption of the cleavage products. This faculty is an adaptation to life on a soil poor in nitrogen compounds. On richer soil the plants can live without capturing animals. Darwin (*282*) was the first to make an elaborate study of carnivorism in plants (1875). In the best-investigated forms (*Drosera, Pinguicula, Nepenthes*) proteolytic enzymes of a catheptic type (*283, 284*), together with an acid, are secreted and accomplish digestion. The prey may be captured by mucus secreted together with enzymes and acid, aided by movements of tentacles and/or leaves (*Drosera, Pinguicula*) or by very ingenious devices: a trap with closing elastic valve in *Utricularia*, a rapidly working trap in *Dionaea*, a kind of complex eel trap in *Genlisea*, a simple pitfall in *Nepenthes* and *Sarracenia* (*284, 285*).

Many species of fungus are able to thrive on protein; some of them have a simple means of capturing their prey: a snare of fungus thread. However, they never make digestion chambers but penetrate into their prey (*285*).

In Darwin's time nothing was known of proteases in plants, so that secretion of enzymes similar to those of the animal stomach and pancreatic juice was as surprising as the many intricate devices by which the animals are captured. At present the development of these morphological adaptations is far more difficult to explain than that of secretion of proteolytic enzymes into a digestive cavity. It is certainly remarkable that some higher plants of a few different families which are not closely related taxonomically, have developed external digestive chambers comparable to the internal digestive cavities of animals.

VI. Concluding Remarks

As far as it has been investigated, the basic pattern of digestion is the same throughout the whole animal kingdom. With the exception of pepsin the same system of enzymes is necessary in all animals, and even plants, for the cleavage of protein to amino acids. All animals have chiefly α-amylases activated by salts (in contrast to plants which have α- and β-amylases not activated by salts) for the digestion of starch and glycogen. Lipases everywhere digest fats and esters.

In vertebrates the digestive enzymes are secreted seriatim in different glands; in invertebrates the enzymes often act in a single digestive juice, or at least the localization is not as pronounced as in vertebrates. The stomach in vertebrates serves as a preliminary store for food and secretes an additional enzyme (pepsin) not found in invertebrates. Both stomach and pepsin are absent in different groups of fishes. The most pronounced localization of enzymes in invertebrate digestion is to be found in the hepatopancreas (mollusks and arthropods with the exception of insects). This organ, often called digestive diverticulum or midgut gland in the English literature, has, besides the secretion of enzymes and absorption, important metabolic functions (transamination, synthesis of various metabolites, storage of glycogen) so that the older name hepatopancreas seems more appropriate.

The most striking differences among the various groups of animals are to be found in the digestibility of cellulose. A limited number of animals (also some vertebrates) are capable of producing cellulase, but its production on a large scale seems to be inefficient. If cellulose is consumed in bulk, the cellulase produced by bacterial symbionts is used for cellulose digestion (ruminants, some marsupials, many invertebrates).

For the digestion of special kinds of food like wax and chitin bacteria are used, although some animals even among the vertebrates are able to produce chitinase. Keratin of wool can be digested after a preliminary reduction which unlocks the —S—S— bridges.

In many animals the food (especially fat) is emulsified in the gut, in vertebrates by means of bile acids, in many invertebrates by emulsifiers that are mostly identified.

Absorption in vertebrates is a polar and "active" process: it is directed from the lumen of the gut to the blood with consumption of oxygen and energy. The same is true for many invertebrates, but in some forms mere diffusion may suffice (at least partly) (*Helix*). Many lower invertebrate groups have retained the faculty of phagocytosis, i.e., the taking up of particles of microscopic dimensions by the cells of the intestinal wall (sponges, sea anemones, lower worms, mussels, *Amphioxus*).

Remarkably, some groups of carnivorous or insectivorous plants have developed devices for capturing small animals and for digesting these in digestive cavities as a compensation for the nitrogen deficiency of their habitat.

ACKNOWLEDGMENTS

The author is indebted to Mr. N. W. H. Houx and Miss E. Vriezen for assistance in collecting recent literature, to Dr. Ch. Jeuniaux for looking through the manuscript, and to Miss S. van Cornewal for redrawing most of the figures.

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CHAPTER 8

Comparative Biochemistry of Detoxification

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I. Introduction 403
II. The Detoxication Mechanisms 404
  A. Oxidations 404
  B. Reduction of Nitro Groups 413
  C. Syntheses and Conjugation Reactions 413
  D. Thiocyanate Formation 433
  E. Hydrolysis 434
  F. Dehalogenations 436
III. Reaction Rates and Species Differences 441
IV. Developmental Aspects of Detoxication 444
V. Detoxication and the Adoption of a Terrestrial Habitat 447
References 448

I. Introduction

Many of the biochemical reactions discussed under this title convert toxic organic compounds to innocuous metabolites, but many other examples are known of "detoxication mechanisms" which considerably increase the toxicity of ingested material. The term remains a useful one, however, and is generally understood to describe those biotransformations that occur when foreign organic compounds not normally found in the organism are metabolized, regardless of whether toxicity is decreased, increased, or remains the same. Of course the increase or decrease of biological activity by a metabolic process is of considerable importance, and when one of the "detoxication mechanisms" discussed below affects this, the terms "activation" or "inactivation" are used.

The chemical changes involved in these "detoxication mechanisms" can be classified mainly as oxidations, reductions, hydrolyses, and syntheses; a full account of these from a chemical and structural standpoint has recently been published (*1*). In the present chapter only those reactions are discussed of which some comparative study has been made.

Probably all living tissue is capable of metabolizing abnormal organic compounds when these are sufficiently like natural metabolites to fit into the normal metabolic systems. Many methylations, oxidations, and hydrolyses of foreign compounds probably occur in this way. In the more highly evolved organisms like terrestrial vertebrates, insects, and higher plants, so-called conjugation mechanisms are also present. These processes, which are detoxication mechanisms par excellence, yield products like sulfuric esters, glucuronides, and hippuric acids, which are almost invariably nontoxic.

The comparative distribution and activity of these mechanisms in different species is one essential factor in the understanding of selective action of drugs and poisons, and the recognition of the importance of detoxication in insecticide-resistant strains has stimulated much investigation of these processes in insects. The similar phenomenon of drug resistance in microorganisms is not, as a rule, associated with detoxication mechanisms (*2*) and only a few examples of drug detoxications by resistant bacteria are known (*3, 4*). Among vertebrates it is now a common practice to investigate the metabolism of a new drug in several of the small experimental animal species, since it is recognized that man may not treat a drug in the same fashion as a particular species. Studies of detoxication in other vertebrates such as birds and reptiles are much less frequent and for aquatic species and invertebrates other than insects, virtually no information is available.

II. The Detoxication Mechanisms

A. Oxidations

1. *Aliphatic Compounds*

The oxidation sequence (I) is frequently found when alkyl substi-

$$R{\cdot}CH_3 \longrightarrow R{\cdot}CH_2OH \longrightarrow R{\cdot}CHO \longrightarrow R{\cdot}COOH$$

(I)

tuted compounds are metabolized in vertebrates and as a rule the process goes rapidly and completely to the acid end product whether the initial or the intermediate compounds are fed. Thus in the usual laboratory mammals, toluene, benzyl alcohol and benzaldehyde are all converted into benzoic acid derivatives (*1*).

This type of oxidation is also found in hens, which excrete aromatic aldehydes as the corresponding acids (*5*). It is also found in frogs and turtles, which oxidize picolines and quinaldine to the corresponding acids (*6*).

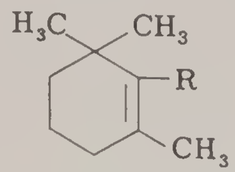
Oxidation of salicyl alcohol to salicylic acid has been observed in insects that consume salicin in their food. *Melasoma*, *Plagiodera*, and *Aromia* adults carry out this oxidation (*7, 8*). In the larvae of the former two beetles, and also in *Phyllodecta* larvae (*9*), the end product is salicylaldehyde, but this is a special case as these larvae secrete the aldehyde in a special repellent gland.

Oxidation of phenylacetaldehyde occurs in many plant saps (*10*), but the oxidation is due to a peroxidase, and benzaldehyde and formic acids are formed as end products. In vertebrates, phenylacetaldehyde is oxidized to phenylacetic acid (*1*).

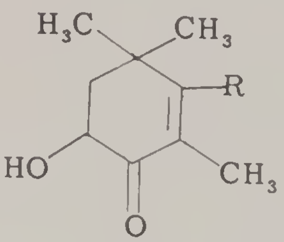
In addition to the terminal oxidation of aliphatic chains to carboxylic acids, vertebrates are also able to introduce hydroxyl groups at other positions in an aliphatic chain to give secondary alcohols. This reaction is a common inactivation process with barbiturate drugs and probably involves a different enzyme from that concerned with terminal oxidation (*11*).

Species differences in the extent of this reaction correlate well with the observed sleeping times of different animals dosed with hexobarbital. Dogs, which slept for 5 hours, oxidized only 36 μg. of drug per gram liver per hour, whereas mice given a similar dose oxidized hexobarbital at about 600 μg. per gram liver per hour and slept for only about 10 minutes (*12*).

Formation of a secondary alcohol by hydroxylation of a foreign organic compound is also found in the oxidation of β-carotene (II) to astaxanthin (III) in the locust (*13*).



(II)



(III)

After formation of a terminal carboxyl group on a long aliphatic chain, the molecule becomes liable to β-oxidation by the normal metabolic processes. The fate of the phenyl fatty acids in vertebrates is well known, and other ω-substituted fatty acids are similarly metabolized by fungi (*14, 15*) and plants as well as by vertebrates. The homologous ω-fluoro fatty acids and amides have alternating toxicities in vertebrates depending on whether β-oxidation can give the toxic fluoroacetic acid (*16*).

Similarly in plants, β-oxidation results in alternating growth-regulatory properties in the ω-aryl- and ω-aryloxyalkylcarboxylic acids (*17, 18*).

Species differences have been observed in the substrate specificity of the β-oxidation process in different plants. Wheat β-oxidizes a wider range of substituted ω-aryloxyalkylcarboxylic acids than peas and tomatoes. In the latter species β-oxidation beyond the stage of the aryloxypropionic or -butyric acid is sterically hindered by substitution of $—CH_3$ or Cl *ortho* to the oxyalkylcarboxylic side chain (*19*).

2. *Aromatic Compounds*

Hydroxylation of aromatic rings occurs in all living material, and many different enzymes and mechanisms are involved (*20*). Many of these enzymes show a high substrate specificity, but the hydroxylation of foreign organic compounds in vertebrate detoxication is carried out by a nonspecific system which seems to be able to hydroxylate almost any aromatic ring. Most of the information on this nonspecific hydroxylation is drawn from experiments with vertebrates, but there is evidence that a very similar system occurs in locusts, in which chlorobenzene is converted to the same mixture of phenols formed by mammals (*21*). It is also possible that insects and many plants metabolize foreign organic compounds with the aid of phenolases. Phenolase activity has been correlated with the detoxication of a series of phenols in fungi by Rich and Horsfall (*22*), who found that in a series of 42 phenols, only those which were not attacked by phenolase were toxic. The colored products of phenolase action were not fungitoxic. Hydroxylations similar to those brought about by the nonspecific vertebrate system also occur in plants: the inactivation of 2,4-dichlorophenoxyacetic acid in bean plants occurs by hydroxylation to 6-hydroxy-2,4-dichlorophenoxyacetic acid, which presumably occurs by an enzyme which is not a phenolase (*23*). The hydroxylation of arbutin to 3,4-dihydroxyphenyl glucoside in bean plants (*24*) may be another example.

The hydroxylation of aromatic rings in mammals is well known, and recent developments have been reviewed (*1*, *25*). Few examples of hydroxylation in birds have been recorded, but Crowdle and Sherwin (*26*), found that nitrobenzene was converted to *p*-aminophenol and Josephson *et al.* (*27*) found that the antimalarial drug pamaquine was metabolized by hydroxylation in chickens. The excreted metabolite, a 5,6-quinoline quinone derivative was a more active antimalarial than pamaquine but more toxic.

Frogs oxidize benzene to phenol (*28*), and frogs and toads convert chlorobenzene to a mixture of phenols similar to that produced by other species (*21*). A metabolite of DDT in flies appears to be phenolic but has not been identified (*29*). Phenothiazine is hydroxylated in the cockroach to a metabolite which occurs also in vertebrates (*30*), but this is not good evidence of a hydroxylase as the leucothionol is also formed spontaneously by oxidation in air.

The orientation of the phenolic metabolites of substituted benzenes is subject to considerable species variation. Aniline was oxidized by a number of vertebrate species to *o*- and *p*-aminophenols, but the relative amounts of *para:ortho* isomer varied in those examined (*1, 31*) from 15:1 for gerbils to 1:2 for cats and dogs. Similar preference for *o*-hydroxylation by cats and dogs has been found with β-naphthylamine. Cats and dogs converted more than half of the dose of this compound to 2-amino-1-naphthol, but rats and rabbits excreted only about 5% of the dose in this form, most of the dose being converted to 2-amino-6-naphthol (*32–34*).

These differences in orientation of the phenolic metabolites of arylamines have excited interest because of the correlation of a high degree of *o*-hydroxylation with the induction of bladder tumors by β-naphthylamine in dogs. A similar situation may exist with 2-acetamidofluorene, which is carcinogenic in rats but not in guinea pigs. Rats excrete about 10% of the compound as 1- or 3-hydroxyacetamidofluorene, but guinea pigs excrete less than 1% of the dose as these *o*-hydroxy derivatives. In cats and dogs, however, acetamidofluorene is not particularly carcinogenic in spite of the general tendency of these two species to hydroxylate arylamines at the *o*-position (*35*).

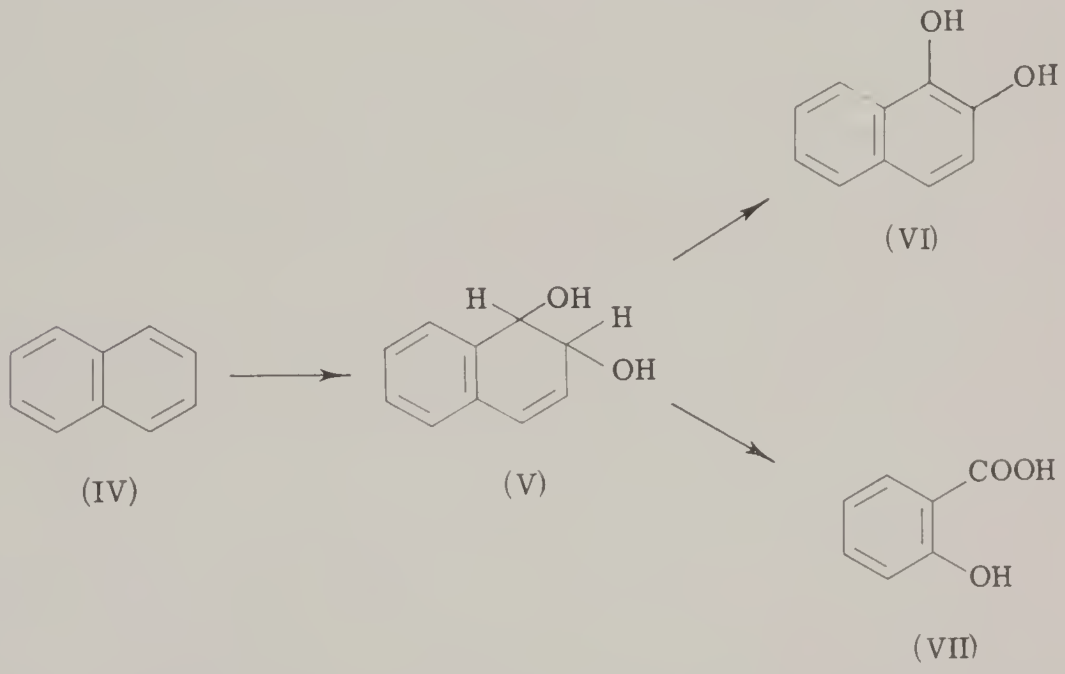
Species variations in the relative amounts of *o*-, *m*-, and *p*-hydroxylation of chlorobenzene by locusts, rats, rabbits, ferrets and cats have been reported, and in contrast to the results with arylamines, cats excreted much more *p*- than *o*-chlorophenol (*21*). From the relative amounts of *m*- and *p*-chlorophenols excreted, it was thought that at least two mechanisms were involved in the formation of urinary phenols.

The variations between species make it difficult to formulate simple general rules for the orientation of hydroxylation, and the orientation in particular cases will depend on the relative concentrations of several enzymes with different specificities.

In addition to the phenolic metabolites of aromatic compounds, dihydrodihydroxydiols may be formed; this reaction is of particular importance in the metabolism of polycyclic hydrocarbons. This type of metabolite has been identified as a metabolite of naphthalene (IV) or substituted naphthalenes in mammals (*36*), flies (*37*), and some soil organisms (*38–40*). The dihydrodihydroxynaphthalenes (V) are capable of existence in two optically active forms; different organisms favor the formation of one or other of these isomers. Rabbits excrete both naphthalene and anthracene as a mixture of (+) and (−) diols in which the (+) isomer is predominant. Rats excrete a mixture containing more of the (—) isomer in both cases. Soil organisms, including *Pseudomonas* and *Nocardia* species appear to form and use only the (+) isomers when adapted to naphthalenes, and only the (—) isomer has been isolated from guinea pig urine.

These results suggest that while the enzyme system in different species is essentially the same, a steric hindrance effect operates, possibly associated with slight differences in the enzyme from different species.

There is evidence that, both in microorganisms and vertebrates, the diols are not intermediate stages in the formation of phenols. The naphthalene-adapted organisms studied by Walker and Wiltshire (*39*) were not adapted to naphthols, and these were not formed in their cultures. Liver preparations as yet have not been shown to dehydrate a diol to a phenol (*41*), but a soluble enzyme is found in liver capable of oxidizing them to the corresponding catechol derivatives (VI) (*42*). The soil organisms converted the diols into salicylic acid (VII).



(IV) (V) (VI) (VII)

Most published examples of hydroxylation of aromatic compounds by fungi and microorganisms do not, strictly speaking, illustrate the metabolism of a foreign organic compound since the organisms are usually adapted to growth on a single source of carbon. Nevertheless the presence of communities of organisms in the soil which can adapt to the presence of an organic compound can lead to the more or less rapid inactivation of such materials. The activity of soil as a whole, therefore, shows some similarity to the detoxicating activity of larger animals and plants and may be of some practical importance.

The herbicide *p*-chlorophenoxyacetic acid is converted by a large number of soil microorganisms to 2-hydroxy-4-chlorophenoxyacetic acid, β-naphthoxyacetic acid is metabolized to β-(6-hydroxynaphthoxy)acetic acid, and phenoxyacetic acid is hydroxylated in the *p*- and *o*-positions by *Aspergillus niger* (*15, 43*).

Hydroxylations so far reported by adapted microorganisms and fungi take place at positions in the benzene ring where the ring could be, or is, attacked by the vertebrate oxidation system. Thus *m*-hydroxybenzoic acid gives gentisic acid and phenol gives catechol (*44*) and aniline gives *p*-aminophenol (*45*) both in some microorganisms and in rabbits. *Nocardia opaca* hydroxylates *o*- and *p*-nitrobenzoic acid *ortho* to the nitro group (*46*). The mechanism in many fungi and bacteria, differs, however, from that in vertebrates since displacement of ring substituents by hydroxyl often occurs. Carboxyl, nitro, and sulfonic acid groups are thus displaced by hydroxyl from benzene derivatives in microbiological oxidations (*44*), but these displacements are almost never found in vertebrates.

In these microbiological oxidations the tendency is for many different benzenoid compounds to be metabolized to a few catechols, such as catechol or protocatechuic acid, which are then opened to give aliphatic 6-carbon compounds. When catechol is cleaved in microorganisms the product is the expected *cis,cis*-muconic acid, but in the analogous reaction in mammals only the *trans,trans*-isomer is excreted (*47*). It is not clear how this inversion comes about, but the ring-opening reaction is a minor one in vertebrates and the muconic acid formed from benzene is poorly utilized. Most of a dose of *trans,trans*-muconic acid is recovered again in the urine.

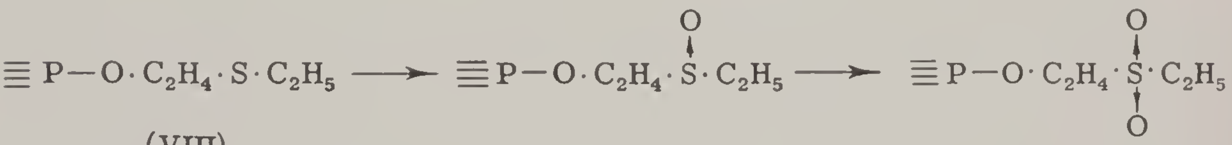
3. *Sulfur Compounds*

Thioether links in foreign organic compounds may be oxidized in tissues to sulfoxides and sulfones. Methylthioaniline is oxidized by mice to the sulfone (*48*). Methylene blue is converted to its sulfone; methylene azure in mammals (*49*) and phenothiazine (*50*) and the related drug chlorpromazine (*51*) are oxidized to the corresponding sulfoxides in mammals.

Phenothiazine is used widely as an anthelminthic in cattle, but after its administration some species may develop corneal opacity due to photosensitization caused by the presence of the sulfoxide in aqueous humor. Phenothiazine is metabolized by most animals to a number of hydroxylated products (*1, 52*), but the blood of calves, sheep, and pigs also contains substantial amounts of phenothiazine sulfoxide, and in calves and pigs this penetrates to the aqueous humor of the eye. At the normal anthelminthic dose sheep are able to metabolize this sulfoxide further and do not become photosensitized, but at higher doses enough sulfoxide accumulates in the eye to cause photosensitization in this species also (*52*).

Other phenothiazine metabolites cause photosensitization if they are injected into aqueous humor, but in practice only the sulfoxide penetrates from the blood, and only in these three species of those studied, is the blood sulfoxide concentration significant enough to cause trouble. Pheasants and goats are also subject to phenothiazine blindness, but the metabolic relationships have not been studied in these species (*52*).

Several organophosphorus insecticides, e.g., Systox (VIII), contain a thioether linkage in the molecule, and this is found to be oxidized to sulfoxide and sulfone in plants, insects, and vertebrates [see reviews by Casida (*53, 54*)] and also in microorganisms (*55*). The oxidation products are usually more easily hydrolyzed than the insecticides and are also more toxic.



$$\equiv P-O\cdot C_2H_4\cdot S\cdot C_2H_5 \longrightarrow \equiv P-O\cdot C_2H_4\cdot \overset{O}{\overset{\uparrow}{S}}\cdot C_2H_5 \longrightarrow \equiv P-O\cdot C_2H_4\cdot \underset{\underset{O}{\downarrow}}{\overset{O}{\overset{\uparrow}{S}}}\cdot C_2H_5$$

(VIII)

Oxidation of the P=S group to P=O in phosphorothioate insecticides (e.g., parathion) is another widely distributed reaction and always produces a marked increase in toxicity. Plants oxidize these compounds more slowly than either insects or mammals, and in their use as systemic insecticides in plants the lethal oxidation normally occurs after the absorption into the insect (*56–58*). Many examples of phosphorothioate oxidation in particular insecticides are quoted in the two reviews by Casida (*54, 55*).

A formally similar desulfuration occurs with thiourea derivatives, e.g., phenylthiourea (*59*), and the toxicity of monosubstituted thioureas has been related to the H_2S formed in the reaction:

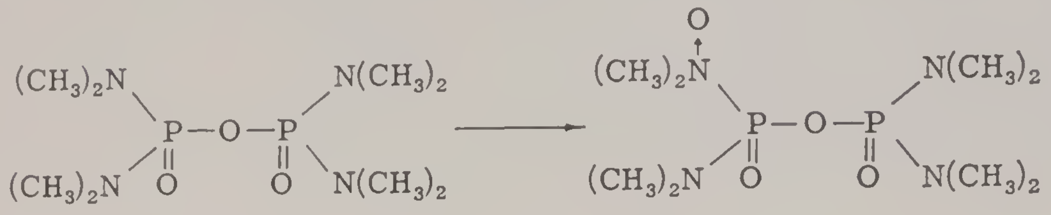
$$R\cdot NH\cdot CS\cdot NH_2 \rightarrow R\cdot NH\cdot CO\cdot NH_2 + H_2S$$

The selective rodenticidal action of naphthylthiourea (*1, 60*) is probably based on the more extensive liberation of H_2S in this reaction by rats.

4. *Oxidative Dealkylations*

Dealkylation of *O*- or *N*-alkylated compounds are carried out in vertebrates by TPNH-dependent oxidative systems which are found in the liver microsomes (*61*) and by TPNH-independent oxidative reactions in the mitochondria (*62*).

The microsomal system is responsible also for the oxidation of the nitrogen atoms in the phosphoramide insecticides, e.g., Schradan (IX),



(IX)

and for the phosphorothioate oxidation (*63*). This insecticide oxidation occurs in plants, insects, and vertebrates and causes an increase in the toxicity of the compound (*53, 54, 56*). It is carried out by an enzyme different from the trimethylamine oxidase of animals (*64*).

N-Oxides are possible intermediates in the dealkylation of *O*- and *N*-alkyl compounds (*65*), and the wide distribution of the phosphoramide oxidation system suggests that oxidative demethylation may also occur widely, though as yet the process has been studied only in vertebrates.

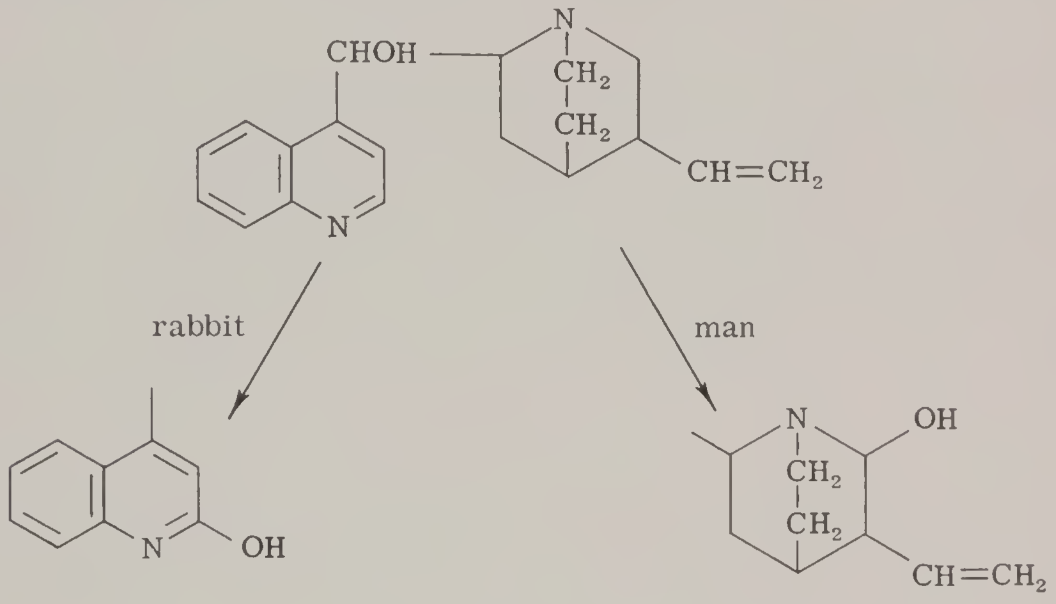
Chickens, pigeons, tortoises, and alligators are able to demethylate *O*- and *N*-alkyl derivatives, but liver microsome preparations are not able to carry out the reaction. Toads (*Bufo marinus*) dealkylate oxidatively, but the enzyme differs from the vertebrate enzyme in being a soluble flavoprotein and not TPNH dependent (*66*). Frogs, salamanders, and four species of fish did not possess a demethylation system (*67*). The metabolism and activation of phosphoramide insecticides by fish does not appear to have been studied, through the presence of trimethylamine oxide in fish is well known.

5. *Epoxidation*

Epoxides have been postulated as precursors of the dihydrodiols formed in naphthalene metabolism (*68*), but the first one observed as a metabolite of a foreign organic compound was the epoxide of heptachlor, which was identified in the body fat after dosing dogs and rats with this insecticide (*69*). A similar conversion of the insecticide aldrin to its epoxide dieldrin, by cockroaches (*70*) and mammals (*71*) has been reported. Heptachlor was also metabolized to the epoxide in flies (*72*). In most cases the epoxides are somewhat more toxic than the precursor. Aldrin and heptachlor are also converted into their epoxides after some weeks on the outside of plants (*73*) and in soils (*74*).

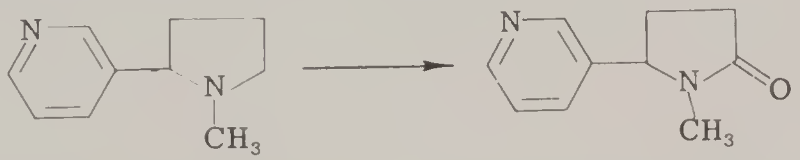
6. *Heterocyclic α-Oxidation*

Hydroxylation of aromatic type heterocyclic compounds is mainly carried out by the same microsomal enzyme which oxidizes other aromatic systems (75). An exception is the quinine oxidase which oxidizes quinine and other heterocyclic nitrogen compounds to carbostyrils. Quinine oxidase is very active in rabbit liver but, with the exception of rats, is of small significance in other species (76). The metabolic path (X) of quinine in rabbits and man is therefore different, the main oxidation in man occuring at the 2-positions (77).



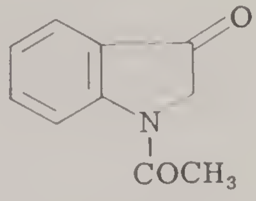
(X)

The same pattern is not followed by nicotine (XI), in which rabbits attack the saturated ring to give cotinine (XII) (78).

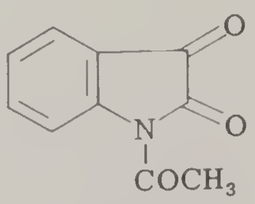


(XI) (XII)

This nontoxic compound is also a major metabolite in the roach (*Periplaneta americana*) (79). An apparently similar reaction occurs in plants, where acetyl indoxyl (XIII) is oxidized to acetylisatin (XIV) (80).



(XIII)



(XIV)

B. Reduction of Nitro Groups

Aromatic nitro compounds may be reduced to arylamines in a wide range of organisms. The reaction is found in mammals (*1*), birds (*26*), reptiles (*81*), and insects (*82*). Reduction of nitrobenzoic acids and drugs containing —NO_2 groups (e.g., Furacin, chloramphenicol) occurs in many microorganisms and may be associated with chloramphenicol resistance in some strains (*46, 83–85*).

Many enzymes are able to reduce the nitro group, and it is probable that a number of normal redox enzyme systems are responsible for the "nitroreductase" observed in tissues. Flavoprotein (*86–88*) and codehydrogenases I and II can be involved in different tissue preparations. Several nitro reducing systems with differing specificities are usually present, and species differences can be referred to differing concentrations of these enzymes (*88, 89*).

The enzymes in microorganisms differ from those in vertebrates in their dependence on cofactors and inhibitors (*85, 89*).

C. Syntheses and Conjugation Reactions

1. *Glycoside Formation*

Foreign organic compounds may be conjugated with a sugar residue in the organism if a hydroxyl, carboxyl, or amino group is present in the molecule to give ether-, ester-, or *N*-glycosides. The resultant β-glycosides usually have a greater water:oil partition ratio than the aglycon, and it is usually found that this results in a detoxication of the original molecule. In animals, excretion is facilitated by the increased water solubility and polarity; in plants, where immediate excretion is not possible, the glycoside accumulates in watery vacuoles in the leaf cells, where it offers less hindrance to the normal biochemical processes of the cytoplasm (*90*), or it is concentrated in other metabolically inactive sites in the plant (*91*).

For historical reasons the classic formation of β-glucuronides by vertebrates is a familiar example of the glycosidic detoxication, but in terms of the number of species involved, β-glucoside formation is probably the most common detoxication reaction of this type. Glucose is used for this reaction by most of the plants so far studied and by all the insect species investigated. β-Glucuronides occur as normal constituents of a few plants, mainly *Scutellaria* species (*92*), but the fate of foreign organic compounds in these plants has not been studied.

A large number of glucuronides or organic compounds has been isolated from mammals (*1, 93*), but the number of species involved is small, most of the data being from rabbits and some results from other laboratory animals. Qualitative and quantitative colorimetric estimations, however, suggest that most mammals, including rats, guinea pigs, mice, sheep, cows, camels, elephants, a number of marsupial species (*1*), and birds (*94*), all can form glucuronides from ingested hydroxylic or carboxylic compounds.

The domestic cat is an exception for it yields negligible amounts of glucuronides when dosed with a variety of glucuronidogenic compounds (*95*). This finding is associated with the lack of the necessary liver enzymes (*96*) and probably accounts for the relatively high toxicity to cats of many organic compounds.

Reptiles are probably also capable of the glucuronide conjugation like other vertebrates, though the evidence is as yet limited. Normal tortoise (*Testudo mauretanica*) urine has been shown by the naphthoresorcinol color reaction to contain glucuronides (*97*), and in the urine of green lizards (*Lacerta viridis*) dosed with 8-hydroxyquinoline, the glucuronide could be identified by chromatographic and ionophoretic techniques (*98*).

Amphibia are also able to synthesize glucuronides. Frogs form a glucuronide on exposure to menthol when well nourished, but not when starved (*99*). When *Rana pipiens* was dosed with phenolphthalein, 8-hydroxyquinoline, α-naphthol, or *p*-nitrophenol, glucuronides, and ethereal sulfates were excreted in a ratio of 4:1, provided glucose was given before the experiment. If this was not done, the ratio of glucuronide to sulfate was 1:5 (*100*). Toads (*Bufo marinus*) and salamanders (*Salamandra necturus*) also formed glucuronides from these phenols. *Bufo bufo* formed the ester glucuronide when dosed with *p*-aminobenzoic acid (*98*). Fish, including goldfish and perch, do not appear to form glucuronides or ethereal sulfates *in vivo* and are highly sensitive to small amounts of phenol in the water (*100*).

In insects, instead of glucuronic acid, the sugar used in detoxication is glucose; and β-glucosides have been identified in insects after dosing with phenols or as conjugates of normally occurring metabolites (see Table I). The β-glucosidase in the gut of insects may in some cases completely hydrolyze the excreted metabolite before it leaves the insect, but whether this modifies the effect of the inactivation process depends on whether phenols can be absorbed from the mid- or hindgut. This is not known, but the few glucosides that have been tested were less toxic than the corresponding phenols when given orally (*81*).

Other invertebrates have received almost no attention. Mussels did not form any glucuronic acid derivative after treatment with borneol or tribromoethanol (*97*); similar attempts to detect glucoside or glucuronide

TABLE I

FORMATION OF β-GLUCOSIDES BY SOME INSECTS

Insect	Aglycon	References
Thysanura		
Lepsima (silverfish)	7-Hydroxycoumarin	*(124)*
Orthoptera		
Periplaneta (cockroach)	Benzoic acid, protocatechuic acid, *m*-aminophenol	*(125, 126, 124)*
Carausius (stick insect)	*m*-Aminophenol	*(124)*
Nomadacris (red locust)	*m*-Aminophenol	*(124)*
Locusta (migratory locust)	Aminonitrocresol, *m*-aminophenol, hydroquinone, 7-hydroxycoumarin, 8-hydroxyquinoline	*(81, 124, 127)*
Schistocera (desert locust)	Aminonitrocresol, *m*-aminophenol, 8-hydroxyquinoline	*(82, 124, 127)*
Coleoptera		
Blaps (cellar beetle)	*m*-Aminophenol, 7-hydroxycoumarin	*(124)*
Coccinella (ladybird)	*m*-Aminophenol	*(124)*
Dytiscus (water beetle)	*m*-Aminophenol	*(124)*
Pterostichus	*m*-Aminophenol	*(124)*
Pyrochroa (cardinal beetle)	*m*-Aminophenol	*(124)*
Tenebrio (meal worm)	*m*-Aminophenol	*(124)*
Lepidoptera		
Smerinthus (poplar hawkmoth)	*m*-Aminophenol, 8-hydroxyquinoline, 7-hydroxycoumarin	*(124)*
Sphinx (privet hawkmoth)	*m*-Aminophenol, 7-hydroxycoumarin	*(124)*
Bombyx (silkworm)	Unidentified	*(128)*
Hemiptera		
Notonecta (water boatman)	*m*-Aminophenol	*(124)*
Aphis	Aphins	*(129)*
Tuberolachnis	Aphins	*(129)*
Diptera		
Drosophila (fruit fly)	*N*-Acetyl-4-hydroxytyramine	*(130)*

TABLE II
β-Glycoside Formation from Foreign Compounds in Some Higher Plants

Plant	Aglycon	Glycoside[c] formed	References
Lentils[a]	*o*-Aminobenzoic acid	G	*(105)*
Wheat germ[a]	Quinol	G, Gb	*(121)*
	Catechol, resorcinol, saligenin	G	*(121)*
Wheat	Phenol	G	*(108)*
Barley	Phenol	G	*(108)*
Maize	Saligenin	G	*(132)*
Corn	Trichloroethanol	G	*(135, 136)*
Tobacco	Trichloroethanol	G	*(135, 136)*
Dandelion	Trichloroethanol	G	*(135, 136)*
Vicia faba (broad bean)	Quinol, resorcinol, catechol, phloroglucinol, saligenin, pyrogallol	G	*(24, 116)*
Vicia faba[b]	Quinol, salicylaldehyde, vanillin	G	*(131)*
Sambucus sieboldiana (mallow)	Vanillin, salicylaldehyde, quinol	G	*(131)*
Mallotus japonicus	Vanillin, salicylaldehyde, quinol	G	*(131)*
Platanus orientalis (plane)	Vanillin, salicylaldehyde, quinol	G	*(131)*
Iris japonica (iris)	Vanillin, salicylaldehyde, quinol	G	*(131)*
Pinus sativa[b] (pine)	Vanillin, salicylaldehyde, quinol	G	*(131)*
Adiantum macrophyllum	Phloroglucinol	G	*(107)*
Pinus resinosa (pine)	Phloroglucinol	G	*(107)*
Briza media (meadowgrass)	Phloroglucinol	G	*(107)*
Pelargonium hortorum	Phloroglucinol	G	*(107)*
Kalanchoe punctata	Phloroglucinol	G	*(107)*
Gerbera jamesoni	Phloroglucinol, quinol, resorcinol, saligenin, vanillin, *p*-hydroxybenzaldehyde	G	*(107)*
Malus sylvestris (apple)	Phloroglucinol, quinol, resorcinol, saligenin, vanillin, *p*-hydroxybenzaldehyde	G	*(107)*

Malus pumilia (apple)	Maleic hydrazide	G	*(133)*
Salix purpurea (willow)	Maleic hydrazide	G	*(133)*
Nicotiana rustica (tobacco)	Maleic hydrazide	G	*(133)*
Nicotiana sanderae (tobacco)	Maleic hydrazide	G	*(133)*
Triticum vulgare (wheat)	Maleic hydrazide	G	*(133)*
Solanum tuberosum (potato)	Chloroethanol	G	*(134)*
Gladiolus sp.	Chloroethanol	G	*(134)*
Gladiolus sp.	*o*-Chlorophenol	Gb	*(109)*
Triticum aestivum (wheat)	Chloroethanol	G	*(134)*
Lycopersicon esculentum (tomato)	*o*-Chlorophenol	Gb	*(109)*
Lycopersicon esculentum	Trichloroethanol	G, Gb	*(102)*
Lagenaria leudantha (gourd)	Trichloroethanol	G	*(135, 136)*
Raphanus sativus (radish)	Trichloroethanol	G	*(135, 136)*

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[a] Extracts with added uridine diphosphoglucose (UDPG).

[b] Extracts with added glucose-1-phosphate and O_2.

[c] G, Glucoside; Gb, gentiobioside.

conjugates in *Helix* or *Arion* after dosing with phenols have been unsuccessful (*98*). Mollusks, however, often contain large amounts of glycosidases, and it is possible that any conjugates formed may have been hydrolyzed again before detection.

The detoxication of excessive amounts of normal metabolic byproducts has long been considered as one function of glycoside formation in plants (*90, 101*). Foreign organic compounds are also converted to glycosides in plants (*102*) (Table II).

In some cases, e.g., maleic hydrazide, a physiologically active compound is inactivated by glucosidation, and it has been suggested (*103*) that the different growth-inhibitory effects of this compound in different species of plants may be related to differences in the extent of conjugation. 2,4-Dichlorophenoxyacetic acid is also probably inactivated in beans and wheat by conversion to a glucoside, from which it may be recovered by treatment with emulsin (*104*). Beans also convert anthranilic acid to an ester glucoside (*105*). The available information is mainly restricted to the higher plants, but the formation of the ester glucoside of anthranilic acid has been confirmed in *Bacillus megaterium*. Only the glucoside was formed, no conjugation with fructose or arabinose occurring when these were supplied (*106*).

The glycoside formed by the detoxication reaction in plants does not necessarily contain the sugar found in the glycoside usually associated with a particular plant. The Solanaceae contain various alkaloid glycosides, such as tomatine from tomato plants, in which the sugar components are usually rare oligosaccharides, but the characteristic detoxication glycoside in this family is a gentiobioside (*102*). This may be compared with the situation in mammals, where the common detoxication sugar is glucuronic acid, but normally occurring *O*-glycosides with other sugars (e.g., galactose in cerebrosides) may be formed for special functions.

The extent of the glucoside or gentiobioside formation in plants may be considerable. In the experiments with dandelions (Table II) the glucoside accounted for 1% of the wet weight of the leaves and 1% of the expressed juice of the tobacco leaf was accounted for by glucoside. In the experiments with *Gerbera* and phloroglucinol (Table II), practically all the C^{14}-glucose given to the leaf was converted to phlorin.

When other sugars were substituted for glucose in the leaf experiments, only glucosides were found: no evidence of any other glycoside was obtained when fructose, galactose, rhamnose, arabinose, xylose, or sedoheptulose were supplied (*107, 108*).

In other plants, where gentiobiosides may be formed, the glycoside produced depends to some extent on the aglycon as well as the species and the site. Chloroethylglucoside was formed from ethylenechlorohydrin in *Gladiolus* corms, but when these were exposed to *o*-chlorophenol, a gentiobioside was isolated (*109*). Miller (*102*) isolated both the glucoside and the gentiobioside of trichloroethanol from the leaves of tomato plants grown in a medium containing chloral or trichloroethanol, but found only the glucoside in the roots.

It was thought possible by Miller that the gentiobiosides might have been secondary metabolites produced by condensation of another glucose molecule with the initial glucoside. This idea is supported by the observation that wheat germ extracts which will transfer glucose to quinol from uridine diphosphoglucose (UDPG) will transfer a further molecule to make quinol gentiobioside (*110*). This gentiobioside formation was not due to any transglucosidase action of emulsin, but was a repetition of the original UDPG transfer reaction involving a separate kinase.

Several mechanisms are possible for the formation of β-glycosides in the detoxication process (*111–114*). One possibility in the transfer of a glycosyl unit from another β-glycoside under the influence of a hydrolytic enzyme like emulsin or β-glucuronidase. In effect, this reaction would not require a source of energy. It has been shown that β-glucuronidase will catalyze the transfer of glucuronic acid from phenolic or menthol glucuronides to a wide range of alcohols (*113*). In these experiments the formation of alcoholic glucuronides was favored by high alcohol concentrations, but it is possible that similar hydrolytic enzymes may have a synthetic role in some situations.

As a rule, the glycoside detoxication requires a source of energy derived from respiring tissue and the activity is lost by homogenizing the tissue. In plants the glucoside conjugation is slowed if the plant has been kept in the dark, i.e., starved, but light is not necessary for the reaction (*107*). The reaction mechanism appears to be essentially the same in microorganisms, plants, insects, amphibia, and mammals. In the respiring tissue an "active" form of the appropriate sugar is formed which has been identified as UDPG in plants and UDPGA in mammals and fish (*115, 116*). The sugar is transferred from this uridine derivative to the acceptor aglycon by a specific transferase in a reaction whose equilibrium lies far over to the glycoside side. Transfer of the sugar from UDPG to phenols has been shown in plants (*110, 117*), bacteria (*118*), and locusts (*98*), and from UDPGA to phenols, alcohols, amines, and carboxylic acids in mammals (*119, 120*). There is evidence that both glucose residues in the plant gentiobiosides come from UDPG in a two-step process (*121*) involving separate specific transferases.

Whether or not an organism will form a particular glycoside depends on the presence of the "active sugar" and the appropriate transferase. Mammalian liver has UDPG, but no glucosides are found in the absence of the glucosyltransferase. Pea plants contain UDPGA but no glucuronyl transferase, so do not form glucuronides. If, however, the transferase from vertebrate liver is added, the pea extracts serve as a source of glucuronic acid for phenol glucuronide synthesis (*122*).

The activity of the UDPG oxidase, which generates the UDPGA in mammalian liver, and the glucuronyltransferase activity have been measured in the livers of trout and cats, which do not produce glucuronides *in vivo*. It was found that cats lacked the transferase enzyme but could oxidize UDPG to UDPGA (*119*). Trout liver on the other hand contained UDPGA and could transfer glucuronic acid to *o*-aminophenol. The trout system was, however, much more heat labile than the corresponding mammalian enzymes, though other, warm water, fish had more stable systems (*116*). This instability may be associated with the stage oxidizing UDPG to UDPGA since Maickel *et al.* (*123*) found no UDPGA in trout and some other freshwater species, though if this was added to the medium the livers of these fish would transfer the glucuronic acid rapidly to suitable acceptors.

2. *Ethereal Sulfates*

A number of different types of hydroxyl groups are found in nature esterified with sulfuric acid, but only the sulfuric esters ("ethereal sulfates") of phenols are concerned in detoxication mechanisms. The esters with nonphenolic hydroxyl are found, for example, in sterol or bile acid sulfates, in carrageenin and chondroitin sulfate, but no instance has been recorded of the conjugation of OH in the foreign organic compound unless it has a phenolic character.

In addition to this phenol conjugation, there is an increasing number of examples of *N*-sulfate or sulfamic acid formation. These sulfamic acids are probably also formed only from aromatic amines, e.g., in rabbits from 2-naphthylamine (*137*); from *p*-aminobenzoic acid in birds and spiders (*98*).

The ethereal sulfate conjugation appears to occur in all mammals (*1*), including several species of whales (*138*), though some species, such as dogs, seem to have a greater capacity for it than others, e.g., pigs (*1*). As well as in mammals, it occurs in birds, reptiles, and amphibia, but not in fish (*139*). Among invertebrates it has been found in some insects and other arthropods, in a few species of mollusk, and in one mold which excretes a characteristic pigment in the form of an ethereal sulfate (Table III).

Fusarubin, a hydroxynaphthoquinone pigment, is present as an

TABLE III
SULFATE CONJUGATIONS IN LOWER ANIMALS

Organism	Compound conjugated	Reference
Reptiles		
Lacerta (green lizard)	8-Quinolinol, *m*-aminophenol	*(98)*
Anguis (slow worm)	8-Quinolinol, *m*-aminophenol	*(98)*
Birds		
Hen	*m*-Aminophenol, phenol	*(150)*
Crow	*p*-Aminobenzoic acid (gives *N*-sulfate)	*(151)*
Amphibia		
Rana (frog)	Phenol	*(152)*
	2-Naphthol phenolphthalein, 8-quinolinol, *p*-nitrophenol	*(139)*
Bufo (toad)	2-Naphthol phenolphthalein, 8-quinolinol, *p*-nitrophenol	*(139)*
Salamandra (salamander)	2-Naphthol phenolphthalein, 8-quinolinol, *p*-nitrophenol	*(139)*
Insects		
Locusta (migratory locust)	*m*-Aminophenol, 8-quinolinol	*(124)*
Schistocerca (desert locust)	*m*-Aminophenol	*(124)*
Nomadacris (red locust)	*m*-Aminophenol	*(124)*
Carausius (stick insect)	*m*-Aminophenol	*(124)*
Blaps (cellar beetle)	*m*-Aminophenol, umbelliferone	*(124)*
Tenebrio (meal worm)	*m*-Aminophenol	*(124)*
Smerinthus (moth, larvae)	*m*-Aminophenol, 8-quinoline	*(124)*
Sphinx (moth, larvae)	Umbelliferone	*(124)*
Notonecta (water boatman)	Umbelliferone	*(124)*
Lepisma (silverfish)	Umbelliferone	*(124)*
Spiders		
Tegenaria (house spider)	Umbelliferone, 8-quinolinol, *p*-aminobenzoic acid (gives *N*-sulfate)	*(98)*
Epeira (garden spider)	Umbelliferone, 8-quinolinol	*(98)*
Lycosa	Umbelliferone, 8-quinolinol	*(98)*
Woodlice	Umbelliferone, 8-quinolinol	*(98)*

ethereal sulfate in *Fusarium solanum* broth. This sulfate is very easily hydrolyzed by cold acid; it is possible that other hydroxynaphthoquinone pigments of this type may also appear as propigments (*140*).

The nature of the prochromogen in the mollusks associated with "Tyrian purple" varies in the different genera which form the pigment, and in some more than one prochromogen is present (*141*). In *Murex trunculus* one of the prochromogens has been identified as indoxylsulfate —the "urinary indican" of vertebrate urine. This is broken down to give indoxyl, which is ultimately oxidized to indigo. Another prochromogen which, as well as indican, is present in *M. trunculus* and which occurs alone in *M. brandaris,* is similar to, but not identical with, 5-bromoindoxylsulfate; this prochromogen ultimately gives rise to dibromoindigo (*141*).

Apart from the half dozen species of mollusk studied in connection with the Tyrian purple problem, there is no record of the sulfate detoxication in mollusks. Attempts to detect this mechanism in *Helix* and *Arion* by the same methods used with insects and spiders were unsuccessful (*98*). It is possible that the aryl sulfatase present in many mollusks (*142*) may decompose any sulfates that are synthesized. It will be recalled that the initiation of research into the arylsulfatases arose from the observation of this enzyme in *Murex* where it might have been unnoticed but for the formation of the indigoid pigment (*143*).

In practically all cases, the ethereal sulfate detoxication deals with only a small part of the administered phenol. Where glucoside or glucuronide formation is available as an alternative conjugation, this usually predominates. This is probably a function both of the rate of formation and of the availability of SO_4^{2-}. In rabbits (*144*) this sets a limit to the sulfate conjugating capacity; for larger doses of phenols, the greater availability of glucuronic acid means that larger doses are excreted mainly as glucuronide.

The enzymatic synthesis of arylsulfates proceeds by two stages. An "active sulfate" is first formed which has been identified as 3-phosphoadenosine-5-phosphosulfate (PAPS) (*145*), and the sulfate is transferred from this to the hydroxylic acceptor by an acceptor-specific sulfokinase. There is evidence that the sugar sulfates, sterol sulfates, and aryl sulfates are formed by separate kinases acting with the same "active SO_4^{2-}," PAPS (*146, 147*). Whether or not a particular species can carry out the sulfate detoxication depends on the presence of a phenol-specific kinase. It is thus found that *Neurospora* and yeasts may synthesize the active SO_4^{2-} but do not form ethereal sulfates as they lack the appropriate transferring enzymes (*148*).

Crude liver preparations can transfer sulfate to both naphthylamine and phenols. Mg^{2+} is required for the amino transfer but not for the phenol transfer (*149*). The kinase does not appear to be the same as any of the widely distributed aryl sulfatases (*142*).

3. *Acetylation*

Acetylation as a detoxication mechanism is concerned only with the acetylation of —NH_2 groups. The acetylation of hydroxyl groups in foreign organic compounds has not been observed. Both aromatic and aliphatic amino groups may be acetylated in the organism, and a few cases are known of the acetylation of the sulfonamide and hydrazide amino groups.

Acetylation of aromatic amines has been known in the common laboratory animals for many years (*1*), but since the advent of the sulfonamide drugs and a convenient method of measuring their acetylation, many other animals have been examined. In most cases the difference between the "free" and "total" diazotizable amine has been taken to be due to acetylated amine. The acetylated derivatives of the sulfonamide drugs are inactive as antibacterial drugs and are often of lower solubility in water (*153*).

The conjugation (acetylation) of sulfonamide drugs and *p*-aminobenzoic acid has been reported in man, mice, rats, rabbits, guinea pigs, cows, horses, sheep, cats, goats, monkeys, and foxes (*154–156*). Among mammals the dog is exceptional in that it does not excrete acetylated aromatic amines in any significant amount. An isolated report of acetylation of sulfadimidine in a dog by Krebs *et al.* (*157*) has been questioned by Marshall (*158*), who found no acetylation of sulfonamides in a number of dogs. Small amounts of acetamido phenols have, however, been observed in S^{35} tracer experiments on the metabolism of aniline and aminophenols in dogs (*159*). On the other hand, dogs can readily acetylate aliphatic amino groups such as occur in the amino acids, and this reaction is probably part of their normal intermediary metabolism involving a different enzyme system (*160*).

When dogs are given acetamido compounds, e.g., acetanilide (*161*), they hydrolyze them to a considerable extent and excrete the free amine. Rabbits, on the other hand, excrete the acetamido compounds (e.g., phenacetin, *p*-acetamidobenzoic acid) mainly unchanged (*162*); it is possible that the difference between the metabolism of aromatic amines in these two species depends as much on the deactylation as on acetylation. Bray *et al.* (*163*) have distinguished two deacetylating enzymes in liver and kidney, one attacking aromatic acetamido compounds and the other specific for aliphatic derivatives. Dog liver contained large amounts of the former and little aliphatic deacylase. The converse was found with rabbits.

Acetylation of the hydrazide group of isonicotinic hydrazide to give nicotinoylacetylhydrazine has been observed in man and rhesus monkeys (*164*) and also in pigeon liver (*165*) and cow liver (*166*). Dogs do not seem to acetylate the hydrazide group in isonicotinoyl hydrazide (*167*).

Birds acetylate aromatic amines (*168*), but the chicken kidney contains an extremely active deacetylating enzyme, so that much of the acetylated sulfonamide measured in fowl blood is broken down again before it appears in the urine. When N^4-acetylsulfanilamide is given to chickens, as much as 75% is hydrolyzed and appears as free sulfanilamide in the excreta (*169*).

In pigeons, on the other hand, deacetylation is less extensive, and about one-third of a dose of *p*-aminobenzoic acid is excreted as a conjugated, presumably acetylated, metabolite, compared with only trace amounts in chicks (*170*). Frogs did not deacetylate acetylsulfanilamide, but fish kidney deacetylated at about the same rate as mammalian species (*169*).

Acetylation of aromatic amines in fish and amphibia is variable; some species do not carry out the reaction (Table IV). In the few rep-

TABLE IV
ACETYLATION OF AROMATIC AMINES IN SOME LOWER ANIMALS

Organism	Arylamine[a]	Degree of acetylation[b]	References
Reptiles			
Lacerta viridis (green lizard)	P	–	(*177*)
Pseudemys elegans (turtle)	ST	Tr	(*178*)
	S, SP, SD, P	–	(*178*)
Amphibia			
Scaphiopus holbrookii (toad)	S, SP, ST, SD, P	+	(*178*)
Bufo valliceps (toad)	S, SP, ST, SD, P	++	(*178*)
Rana pipiens (frog)	S, SP, ST, SD, P	–	(*178*)
Rana catesbiana (frog)	S, SP, ST	–	(*178*)
Hyla cinerea (tree frog)	S, SP, ST, SD	+	(*178*)
Bufo bufo (toad)	P	+	(*98*)
Insects			
Locusta migratoria (locust)	P, ANC	++	(*98, 179*)
Bombyx mori (silkworm)	P	++	(*180*)
Galleria mellonella (waxmoth)	P	++	(*180*)
Fish			
Squalus acanthias (dogfish)	S	+	(*168*)
Sculpin	S	+	

[a] Abbreviations: P = *p*-aminobenzoic acid; S = sulfanilamide; SP = sulfapyridine; ST = sulfathiazole; SD = sulfadiazine; ANC = 6-amino-4-nitro-*o*-cresol.

[b] Symbols: †, approximate extent of acetylation; –, not detectable; Tr, ca. 0–5% of dose; +, ca. 5–20% of dose; ++, ca. 20% of dose.

tiles which have been examined, the reaction does not take place to any significant extent (Table IV) though whether this is owing to lack of synthetic ability or to a kidney deacetylase, as in birds, has not been considered.

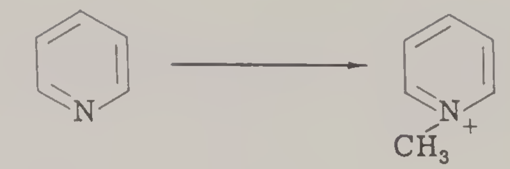
The reaction has been observed in some insects (Table IV). Silkworms had a limited capacity for acetylating *p*-aminobenzoic acid so that smaller percentages of larger doses were conjugated. The percentage conjugation also depended on the rate of feeding and fell rapidly in starvation. A consequence of this was that whereas a high conjugation (60% of the dose) was observed between molts, the cessation of feeding before molting caused a fall to about 15% of the dose, and near the start of cocoon spinning no conjugation at all was seen. It seems possible that in insects where short periods of intense biosynthetic activity occur, as at molting or cocooning time, the detoxication mechanism may be starved of the normal supply of acetate. Deacetylation of acetylarylamines has also been observed in several economically important insect species (*171, 172*).

Some plants also acetylate sulfanilamide. It is absorbed by the roots of broad beans (*Vicia faba*) and acetylated on either the amino or sulfonamide group so that N^1- and N^4-acetylsulfanilamide as well as some N^1,N^4-diacetyl derivative appears in the leaves. A slow deacetylation occurs in leaves and stems, but 30% of the adsorbed material was found in an acetylated form. Similar acetylations and deacetylations occurred, though to a smaller extent, in wheat plants (*173*). Phenylhydrazine is acetylated by respiring yeast (*174*).

The mechanism of the acetylation reaction has been established in mammals and birds and is similar in general form to the other conjugation reactions (*175*). The mechanism in cockroaches (*Periplaneta*) and flies (*Musca*) is probably the same (*176*). The acetate used in the conjugation is "activated" by conversion to acetyl coenzyme A. This may be used in many reactions in the cell such as oxaloacetate formation, acetylating amino acids or glucosamine, if the appropriate acetyl-transferring enzyme is present. If an arylamine acetyl kinase is present, acetyl is transferred to the aromatic amine in the detoxication reaction.

4. *Methylations*

Methylation of amino groups is a normal reaction in many plants and animals, but in foreign organic compounds this reaction occurs most commonly at a heterocyclic nitrogen atom. Thus pyridine and quinoline rings are converted to quarternary ionic compounds in most mammals (*1*), in frogs and turtles (*181*), in hens (*182*) (XV), and probably in some insects (*183*). *N*-Methyl pyridinium hydroxide is also a normal



(XV)

constituent of crabs, mussels, and actinozoans, but the fate of foreign compounds in these has not been studied. These highly ionized metabolites are usually less toxic than the original heterocycles, probably because they are readily excreted by the vertebrate kidney (*1*).

The capacity for this reaction is limited in most animals and larger doses of, for example, nicotinic acid, are mainly handled by other detoxication reactions, like glycine conjugation (*1*). In general, urines of herbivores and ruminants contain less of the methylated metabolites if other routes are possible though the relative amounts of methylated and other metabolites depends to some extent on the dose (*184–186*).

This generalization does not extend to insects, as larvae of neither the carnivorous fleshfly *Lucilia* or the herbivorous moths *Bombyx* or *Corcyra* excrete any methyl nicotinamide (*187, 188*). This compound has been detected in the excreta of the flour beetle *Tenebrio* (*183*).

Some hydroxyl groups, mainly in hydroxy aromatic acids, may also be methylated in man and rabbit, and in man the methylation of the *m*-hydroxyl group is the chief mode of deactivation of physiological amounts of adrenaline (epinephrine) (*189*).

Methoxyl and methylamino groups are common in plant material and the methylation of both phenolic and carboxylic hydroxyls of *p*-hydroxycinnammic acid has been directly demonstrated in the wood-destroying mold *Lentinus lepideus* (*190*).

Methylation of thiol groups also occurs in vertebrates and the methyl thiol may be further metabolized to sulfoxides or sulfones (*1, 191, 192*). The same reaction occurs in the mold *Scopulariopsis brevicaulis* (*191, 192*), which reduces dialkyldisulfides to thiols and then methylates the thiol. Thus a series of dialkyldisulfides from methyl to *n*-amyl were converted to the corresponding methylalkylsulfides.

Several molds (*Penicillium, Aspergillus, Scopulariopsis, Mucor* spp.) reduce and methylate alkylarsonic acids. Thus cacodylic acid $(CH_3)_2 \cdot AsO \cdot OH$ is converted to trimethylarsine and propylarsonic acid, $C_3H_7 \cdot As \cdot O(OH)_2$, to dimethylpropylarsine (*191*). Pentavalent arsenicals are reduced to trivalent arsenicals in vertebrates (*1*), but there is as yet no evidence of methylation of these compounds in the higher animals.

Inorganic sulfur, selenium, and tellurium compounds are also reduced and converted to volatile dimethyl derivatives in molds (*191*). Similar volatile tellurium and selenium compounds are excreted by vertebrates and, in the case of selenium in rats, the compound has been identified as dimethylselenide (*193*).

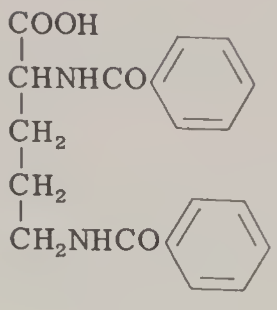
5. *Peptide Conjugations of Aromatic Acids*

Aromatic compounds containing a carboxyl group can be eliminated from the organism as glycosides (e.g., benzoyl glucuronide), but in most animals an alternative detoxication mechanism in conjugation with an amino acid. Glycine is by far the most frequently used amino acid but glutamine, ornithine, and serine are also encountered. The occurrence of these less common amino acids depends to some extent on the acid being metabolized as well as on the species, and two amino acids may sometimes be used simultaneously.

As a rule glycine conjugation predominates in the herbivorous animals, though the only carnivores studied are dogs and cats. Some degree of glycine conjugation is found in all cases, and species known to form hippuric acid include man, dogs, cats, rats, rabbits, mice, calves and cows, goats, sheep, pigs, monkeys, guinea pigs, horses (*1*), camels (*194*), and elephants (*195*).

A serine conjugate of xanthurenic acid is formed by rats (*196*), but this, so far, is an isolated example. The use of glutamine for conjugation depends both on species and the acid administered. Conjugation with glutamine was first observed in man in the metabolism of phenylacetic acid (*197*); phenylacetyl glutamine was also formed by the chimpanzee, but not by other primates, which gave the normal glycine conjugate (*198*). A number of substituted phenylacetic acids tested did not give rise to glutamine derivatives (*197*) in man, but indoyl-3-acetic acid is excreted by man as a glutamine derivative (*199*). *p*-Aminosalicylic is said to form a glutamine conjugate in rats, rabbits, and chickens (*200*), but other workers have been unable to confirm these observations and have found the usual glycine conjugation of carboxyl group in man, rabbit, mice, rats, and dogs (*201*).

If conjugation of this acid with glutamine in the chicken does occur, it is exceptional: it has not been found with any other aromatic acid. Phenylacetic acid is excreted by hens as diphenacetylornithine (*202*). There are some indications that well-fed chickens can form hippuric acids (*203*); nicotinuric acid has been found in chick excreta after feeding nicotinamide (*204*). In most cases, however, hens form ornithuric acids when dosed with aromatic acids (*1*). It is usually assumed that the formation of ornithuric acid (XVI) from benzoic acid is a biochemical characteristic of birds, but the evidence is almost entirely confined to hen experiments, though some tissue experiments (*205*)



(XVI)

suggest that ducks and pigeons might form ornithuric and hippuric acids, respectively. Analytical figures for ether-insoluble diazotizable amine excreted by pigeons have been interpreted by Efimochkina (*170*) as a measure of ornithine conjugation, but in the light of more recent work (*206*) her figures probably represent a combination of *p*-aminohippurate and *p*-aminobenzoyl glucuronide.

A recent study of the conjugation of benzoic acid in a number of bird species (*206*) shows that the ornithine conjugation is not general in birds (Table V). Pigeons and doves dosed with benzoic acid excreted hippuric acid and benzoylglucuronide, but no ornithuric acid was found. Salicylic acid gave similar results (*98*). All avian species examined formed benzoylglucuronide, but in many cases neither ornithuric acid or hippuric acid formation could be detected (*206*). Pigeon tissue extracts have been shown to conjugate benzoic acid with glycine (*205*) *in vitro.* No activity was shown toward ornithine.

The ornithine conjugate excreted by hens in urine is α,δ-dibenzoylornithine, and monobenzoylated ornithines are found only in small amounts, if at all, in catheterized urine. If the urine comes in contact with feces, however, bacterial contamination results in hydrolysis and ultimate destruction of the ornithuric acid (*206*). N^5-Monobenzoylornithine was detected as an intermediate in the destruction, and it is probable that the N^2- and N^5-nicotinoylornithines found in chick excreta after dosing with nicotinamide (*204*) are similar artifacts.

Reptiles, which have a close phylogenetic relationship to birds, are also capable of the ornithine conjugation, though in the species so far examined, the glycine conjugation is also found. Glycine conjugates of benzoic acid and phenylacetic acid were found in the urine of a turtle after dosing with these acids or γ-phenylpropionic acid (*181*). Some analytical figures for the hippuric acid in normal urine of a number of reptiles have been quoted by Khalil (*207–210*), but the method was not specific; probably the figures represent the sum of hippuric and ornithuric acids present.

In the lizard *Scinus officinalis*, however, no hippuric acid was found,

TABLE V

AMINO ACID CONJUGATIONS IN SOME LOWER ANIMALS

Organism	Acid	Conjugates formed[a]	Reference
Insects			
Aedes aegypti (mosquito)	C^{14}-Benzoic	G	*(213)*
Bombyx mori (silkworm)	3-Hydroxyanthranilic	G	*(214)*
Bombyx mori	Anthranilic	G	*(215)*
Locusta migratoria (locust)	Benzoic, *p*-aminobenzoic, *p*-nitrobenzoic, salicylic	G	*(232)*
Corcyra cephalonica (rice moth)	Nicotinic	G	*(188)*
Musca sp. (housefly)	Benzoic, phenylacetic	G	*(233)*
Reptiles			
Natrix natrix (grass snake)	Benzoic, *p*-aminobenzoic	O, some G	*(177)*
Lacerta viridis (lizard)	Benzoic, *p*-aminobenzoic, *p*-nitrobenzoic	O, some G	*(177)*
Anguis fragilis (slow worm)	Benzoic	O	*(177)*
Caiman sp. (alligator)	*p*-Aminobenzoic	O, G	*(177)*
Testudo graeca (tortoise)	Benzoic, *p*-aminobenzoic, salicylic	O, G	*(177)*
Emys europaeus (terrapin)	Benzoic, *p*-aminobenzoic	O, G	*(177)*
Turtles	Benzoic, phenylacetic	G	*(181)*
Amphibia			
Frogs	Benzoic,	G	*(211)*
Frogs	Picolinic	G	*(212)*
Birds			
Hens	C^{14}-Benzoic	O	*(206)*
Chicks	Nicotinic	O, G	*(204)*
Pigeons	C^{14}-Benzoic	G	*(206)*
Wood pigeons	Benzoic	G	*(206)*
Barbary doves	Benzoic	G	*(206)*
Turkeys	Benzoic	O	*(206)*
Ducks	Benzoic	O	*(206)*
Geese	Benzoic	O	*(206)*

[a] O, ornithine conjugates; G, glycine conjugate.

and in *Chalcides ocellatus* the amount formed less than 0.2% of the urine nitrogen (*209*). Urine from tortoises dosed with aromatic acids was found to contain conjugates with both glycine and ornithine (Table V); similar results were found with a grass snake, two species of lizard, and an alligator (*177*).

Formation and excretion of these metabolites was slow, about a third of the dose being excreted in a week, mainly in the conjugated form. Alligators and tortoises formed roughly equal amounts of glycine and ornithine conjugates, but lizards and snakes formed only small amounts of the hippuric acids, and the ornithine conjugates preponderated.

In reptiles, urine and feces may mix in the cloaca and, as with hens, fecal contamination of the urine leads to a decomposition of the conjugates present. The small amounts of N^5-*p*-aminobenzoylornithine found in some reptile excreta (*177*) was probably an artifact.

Frogs are reported to form hippuric acid from benzoic acid and phenylacetic acid (*181, 211*). Picolinuric acid has also been isolated from frogs dosed with picoline or picolinic acid (*212*).

Glycine conjugation has been observed in a few insects in response to administration of foreign acids or as a consequence of the accumulation of a normal metabolite (Table V). Mosquito larvae grown in a medium containing benzoic acid convert some of this to hippuric acid, which is less toxic (*213*). A white mutant silkworm strain which lacked the ability to form pigment from 3-hydroxykynurenine accumulated 3-hydroxyanthranilic acid, which was then conjugated with glycine (*214*). Another strain which could not hydroxylate kynurenine degraded this to anthranilic acid, which was excreted as its glycine conjugate (*215*).

Other invertebrates do not appear to have been investigated apart from an early, somewhat doubtful identification of hippuric acid in the Kebers' gland of a mussel (*216*).

Detoxication of aromatic acids in plants probably occurs only by glycoside formation, but it has been stated (on the basis of analytical results only) that nicotinuric acid is formed in germinating beans (*217*).

The amino acid conjugation occurs in the liver and kidney except in dogs and chickens, where the kidney is the only site (*218*). Conjugation of benzoic acid and phenylacetic acid in mammalian liver involve the initial conversion of the acid to benzoyl CoA or phenylacetyl CoA (*219–221*), and the enzyme activating the acid is probably the same as that catalyzing the condensation of CoA with octanoate (*222*). The acid is then transferred enzymatically to a glycine or glutamine molecule. An enzyme, glycine *N*-acylase, which can catalyze this transfer and form hippuric acid has been separated from beef liver (*223*), but in liver slices or crude preparations the reaction does not always give the same products as in the whole animal since the enzyme can transfer other acyl residues to glycine from their CoA derivatives. In experiments with chicken and duck kidney preparations, the enzyme would transfer both acetyl and valeryl groups to ornithine (*205*). Chick kidney preparations could also form lysine derivatives of aminobenzoic acid if the former amino acid was supplied instead of ornithine, though lysuric acids are not found in the whole animal (*170*).

Human liver, but not that of rat or cow, transferred phenacetyl groups from phenacetyl CoA to glutamine (*221*), and rat or beef liver mitochondria fortified with the kinase from human liver could also form phenacetylglutamine. The synthesis of other conjugates in tissue preparations is suggested by the short report of Klingmuller and Strohmeyer (*224*), who found that their liver preparation could form hippuric acid if glycine, glutamic acid, glutamine, or asparagine was supplied. Phenylacetic acid and *p*-aminosalicylic acid formed both glutamine and asparagine derivatives.

The site of these conjugations inside the liver and kidney cells is in the mitochondria (*225*) in contrast to many other detoxication reactions which are found in the microsomes. It is significant that this is one of the few detoxications which is applied to a molecule already ionized at body pH and that mitochondria can absorb ions from solution (*226*) whereas the microsomes appear to deal primarily with nonionized material. This detoxication enzyme is different from that conjugating the bile acids with glycine since the latter occurs in the microsomes (*227*).

All terrestrial animals appear to have to some degree the ability to form glycine conjugates of foreign aromatic acids; the main comparative interest is in those animals—birds and reptiles—which have the additional ornithine conjugation. Modern birds and reptiles are thought to have evolved from a group of ancestral reptiles different from those which gave rise to the mammals. Amphibia, which branch off the evolutionary tree at an earlier stage, apparently have only the glycine conjugation, so presumably the use of ornithine for detoxication appeared about 200 million years ago in the branch of reptiles which gave rise to the modern reptiles and birds. The bile acids do not appear to be conjugated with either glycine or ornithine in birds and reptiles (*228*).

There is an apparent relation between uricotelic nitrogen metabolism and the possession of the ornithine conjugation, and it is significant that in hen's eggs the first appearance of this conjugation is at about the time the egg starts to synthesize large amounts of uric acid. It has been suggested (*177*) that the use of uric acid, where four of the carbons come directly or indirectly from glycine, as an end product of nitrogen metabolism may have made the existence of an alternative detoxication mechanism an evolutionary asset. It may be recalled also that glycine is a semiessential amino acid in the chick.

Among the few reptiles studied it is the more highly evolved, which also have the most uricotelic nitrogen metabolism, that use mainly ornithine in detoxication. The more primitive groups, turtles and the alligator, have a more "primitive" nitrogen metabolism, partly uricotelic and partly ureotelic, and retain to a considerable extent the glycine conjugation of their amphibian ancestors.

Although the ornithine conjugation is associated with uric acid as a nitrogenous end product, the converse is not necessarily true, as shown by the example of the pigeon. Insects also are usually completely uricotelic and synthesize their uric acid from the same intermediates as birds (*229, 230*), but still use a glycine detoxication.

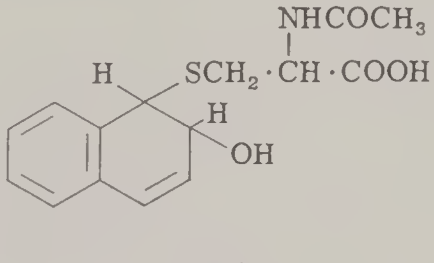
Glycine is also an essential amino acid in *Aedes aegypti* (*231*) despite the use of this amino acid by the mosquito for detoxication.

Regarding *Bombyx mori* and *Musca* sp., see Table V (*232, 233*).

6. *Cysteine Conjugations*

Mercapturic acids are formed by replacement of an atom or group in the foreign organic compound with an *N*-acetylcysteine residue. These derivatives have been detected as metabolites of organic compounds in man, dogs, cats, rabbits, rats, mice, guinea pigs (*1*), locusts (*234*), and flies (*235*).

Two types of organic compounds are metabolized to these cysteine derivatives. In one type, a halogen (e.g., in benzyl bromide, dinitrochlorobenzene) or a nitro group (e.g., pentachloronitrobenzene) is replaced; and in the second, a hydrogen atom in an aromatic ring is substituted (*1*). Mercapturic acids from the second type of compound are excreted as acid-labile precursors containing a partially reduced aromatic ring.



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One of these (XVII) has been isolated from rabbit urine as a metabolite of naphthalene (*236*) and was also present in urine of rats, mice, hamsters, guinea pigs, and man after dosing with naphthalene. Similar premercapturic acids have been detected in the urine of rats and rabbits dosed with benzene, anthracene, or halogenobenzenes (*237*) and in the excreta of locusts dosed with chlorobenzene (*234*).

The cysteine for the mercapturic acid synthesis is of endogenous origin (*238*); the immediate source is probably glutathione (*239*). When S-substituted glutathiones are given to animals the corresponding mercapturic acids are excreted (*240, 241*). It has been shown that rat liver forms S-(1,2-dihydro-2-hydroxyl-1-naphthyl)glutathione, which is converted to S-(1-naphthyl)glutathione on acidification. The labile precursor or the naphthylglutathione were both converted to the corresponding cysteine and then acetylcysteine derivatives by tissue homogenates (*242*).

The process of formation of the cysteine conjugates by insects is probably similar to that in mammals, since in the cockroach bromoacetic acid is rapidly converted to a material which is ionophoretically identical with S-(carboxymethyl)glutathione (*243*). Cysteine derivatives of bromo- or chloroacetic acid have not so far been reported in vertebrates, but aliphatic halogen compounds, e.g., *n*-propyl bromide are excreted by rabbits as mercapturic acids (*244*).

In mammals the excreted metabolites contain an acetylated cysteine residue and the unacetylated compounds have not been reported as urinary products, though such compounds are known to occur as intermediates in tissue experiments (*245*). In locusts, on the other hand, most of the cysteine derivatives excreted were not acetylated; this result did not seem to be due to the action of a deacetylase since injected *p*-chlorophenylmercapturic acid could be recovered in the excreta after injection (*235*).

Cysteine derivatives or mercapturic acids are probably produced as metabolites of some chlorinated insecticides in insects. Bradbury and Standen (*246*) have found that dichlorobenzenethiols, corresponding to over 60% of the dose, are products of the alkaline hydrolysis of metabolites of α- or γ-hexachlorocyclohexane in flies. These were probably derived from the cysteine-containing metabolites.

Water-soluble metabolites were formed from hexachlorocyclohexane by an enzyme preparation from flies in the presence of glutathione, but not in its absence (*246*). Glutathione is also required as an essential cofactor in the action of DDT dehydrochlorinase in the fly, but there is, as yet, no evidence for the formation of any cysteine metabolites of DDT.

D. Thiocyanate Formation

Small doses of cyanide may be detoxified in the organism by conversion to a variety of 1-carbon compounds and to 2-iminothiazolidine-4-carboxylic acid or to thiocyanate (*1*). The rhodanese system, in which cyanide reacts with thiosulfate to yield thiocyanate is quantitatively the most important system in most animals, and the distribution of this enzyme has been the subject of comparative study. This enzyme, which in mammals is found in the mitochondria of the cells, occurs widely in nature from man to bacteria; highest concentrations are found in vertebrate liver and kidney, frogs being particularly rich in the enzyme (*247, 248*). Rabbits, rats, cows, man, and monkey contain less, and the dog least of all among the usual laboratory animals (*248, 249*). Dogs, however, have sufficient enzyme to cope with the detoxication of 4–5 kg. of CN^- in 15 minutes, a quantity so much in excess of the lethal dose of cyanide that detoxication as a normal physiological role for rhodanese has been questioned (*250*).

Rhodanese has been found in cats, horses, sheep, goats (*251*) and hens (*252*), in addition to the vertebrates mentioned. Small amounts were found in the fishes, cod, mullet (*253*), and *Trachurus* (*254*). Activity in the squid was very high (*255*), and small amounts have been found in the intestinal parasites *Taenia* and *Ascaris* and also in liver flukes (*256*). The only insect mentioned is the larva of *Gasterophilus equi,* which contains small amounts of rhodanese (*256*).

The enzyme is found in plants; as the thiocyanate-containing plants beet, cabbage, and nettles do not contain rhodanese, it cannot be a source of their thiocyanate (*250*). *Secale cercale, Avena sativa, Hordeum vulgare,* and *Triticum vulgare* have been found to contain small amounts of rhodanese, but it was not detected in *Triticum spelta, Medicago sativa, Trifolium pratense,* or *Onobrychis sativa* (*257*).

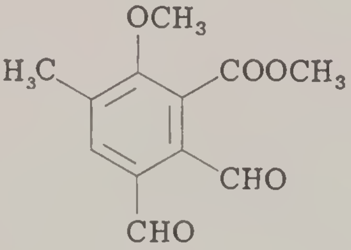
E. Hydrolysis

Many different esterases and lipases are found in all organisms, and hydrolysis of ester links in foreign organic compounds is a common detoxication mechanism. As substrate specificity of these enzymes is often low, a foreign organic compound may be hydrolyzed by several different enzymes. It is difficult to forecast to what extent a given species will hydrolyze a particular foreign compound, but it is found empirically that introduction of an ester link into a toxicant often enables a useful selective action to be obtained. Many drugs, pesticides, and crop control agents are inactivated by hydrolytic reactions.

Comparative hydrolysis rates are a factor in the inactivation of pyrethrins, and a major factor in their low vertebrate toxicity and low oral toxicity in insects is the hydrolysis in the gut (*258*). Pyrethrins are also inactivated by hydrolysis after absorption through the integument of insects; small amounts of chrysanthemumic acid has been identified as a metabolite in flies though other unidentified products were also formed (*259, 260*).

The activity of piperonyl butoxide as a pyrethrin synergist in insects is, to some extent, due to inhibition of hydrolysis (*261, 262*). While the small esterase activity can be inhibited *in vitro* by pyrethrin synergists, their function in whole insects is less well defined and the idea of esterase detoxication of pyrethrin has been contested (*263*). Pyrethrins are probably metabolized mainly by oxidative processes in insects (*54, 264*); the effect of piperonyl butoxide on these as yet unidentified reactions still requires investigation.

Esterase activity does not always lead to inactivation, and it is sometimes convenient to use a neutral ester derivative which is easily absorbed and is hydrolyzed to an effective agent in the organism. This is probably the basis of the fungicidal action of the esters of pentachlorophenol (*265*). Acetylgladiolic acid (XVIII) also owes its fungistatic activity to hydrolysis of the ester group in *Botrytis allii* (*266*). The selective fungitoxic action of 1,4-dimethoxynaphthalene on *Stemphylium sarcinaeformae* is due to its hydrolysis in this species, but the resistant *Monilinia fructicola* lacks the esterase (*267*).



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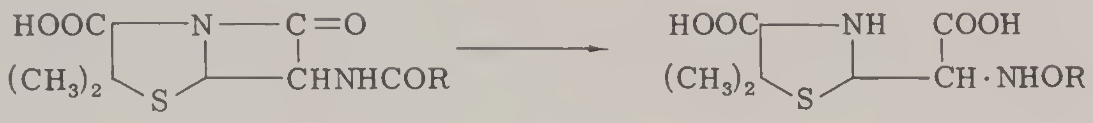
Hydrolysis of the organophosphorus insecticides by phosphatases, amidases, or esterases may occur in plants, insects, and animals and gives rise to essentially nontoxic products (*53*). This phosphatase action is usually preceded by other metabolic changes that may result in activation.

Many of the esterases are poisoned by organophosphates; a mixture of two organophosphate insecticides may have a much greater toxic effect than the sum of their individual activities if their inactivation is normally brought about by esterases. The dangers of the synergistic action of trace amounts of such insecticides in foods has been emphasized by DuBois (*268*).

Amides are readily hydrolyzed in most mammals and excreted as the corresponding acid or conjugate of this (*1*). Penicillin is metabolized in animals to penicilloic acid by hydrolysis (XIX) of an amide link and by removal of the acyl group COR (e.g., as phenylacetic acid with benzylpenicillin) (*1*).



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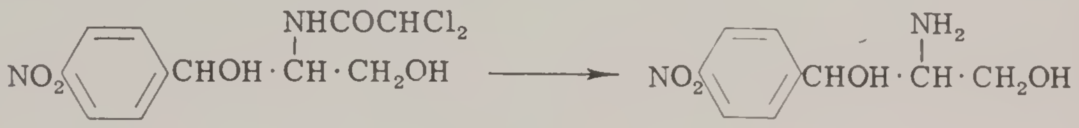
Several penicillin-resistant staphylococci and *Escherichia coli* also hydrolyze penicillin to the inactive penicilloic acid by the penicillinases (*269*).

Amidase action (XX) also occurs in the metabolism of chloramphenicol in mammals (*270*) and in a number of microorganisms (*271*); the deacylated product is not bactericidal.



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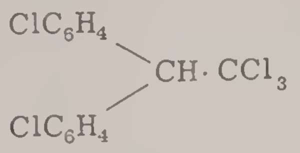
F. Dehalogenations

Four kinds of dehalogenation of halogen-carbon compounds may be distinguished in living systems. The halogen atom may be replaced by a cysteine residue (see above), by hydroxyl, or by hydrogen. In addition, the elements of hydrogen halide may be removed to yield an unsaturated compound.

Defluorination of the fluorophosphonate insecticides, e.g., diisopropyl fluorophosphonate, occurs widely in mammals and in microorganisms. The enzyme responsible for this detoxication process has been identified with an *N*-acetylamino acid acylase (*272*).

Reductive dehalogenation (i.e., replacement of Cl by H) of aromatic chlorine compounds has been shown to occur to a small extent in several mammalian species and also in locusts, where chlorobenzene is metabolized to phenol (*21*). Similar dechlorinations of polychlorobenzenes to give chlorophenols with fewer chlorine atoms also occurs in rabbits (*273*), but it is not certain whether these dechlorinations occur in the animals or whether they are carried out by the microorganisms in the gut. It is also possible that they are a consequence of the mechanism of biological hydroxylation (*21, 273*). Reductive dehalogenation is also carried out by soil bacteria; the destruction of chlorophenoxyacetic acid herbicides in soils (*44*) involves the removal of Cl^- from the chloromuconic acid formed at an intermediate stage of the metabolic sequence.

Hydrolytic dechlorination appears so far to have been observed only in vertebrates. In the case of DDT (XXI), this reaction gives DDA (XXIII) either directly or via DDE (XXII), a possible intermediate formed by dehydrochlorination.



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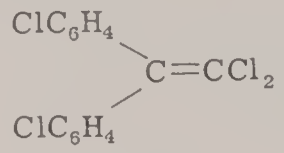

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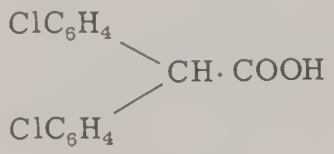

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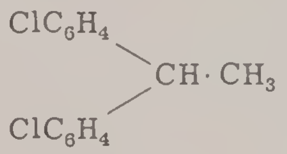
In spite of considerable work on the metabolism of DDT, DDA has not been identified as a metabolite of DDT in insects, and it may be that insects do not have a hydrolytic dechlorination system. The related compounds DMC (XXIV) and DME (XXV) are rapidly converted to a metabolite which is probably DDA (*274*), but this appears to be the result of an oxidative process.



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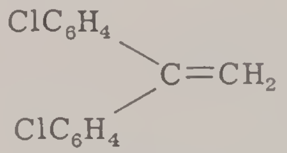
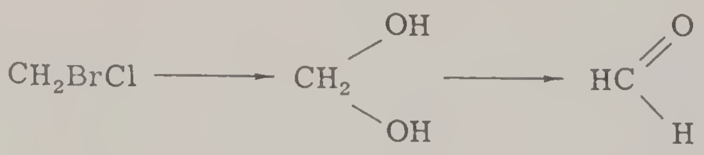


(XXV)

Other examples of dechlorinations in the common laboratory animals are cited by Williams (*1*), but mention may be made of the study by Heppel and Porterfield (*275*) of the hydrolytic dechlorination of some simple aliphatic halogen compounds by rat tissues. The liberation (XXVI) of formaldehyde was facilitated by the presence of reducing agents such as CN^-, cysteine, and glutathione. In contrast to hydrolytic dechlorination, the dehydrochlorination process has been observed in both insects and mammals. Gammexane (γ-hexachlorocyclohexane) is thus degraded both by insects and mammals by loss of the elements of HCl, although the enzyme system is probably different from that attacking DDT (*276*). Pentachlorocyclohexene has been detected as a minor metabolite of gammexane in flies (*276, 277*), and in rabbits the α-isomer



(XXVI)

of benzene hexachloride in excreted to a small extent as 1,2,4-trichlorobenzene (*278*). Investigations of the tainting of vegetables by gammexane have shown that carrots also dehydrochlorinate gammexane to a metabolite which is probably pentachlorocyclohexene (*279*).

Although DDT and some similar insecticides are metabolized by other reactions as well as dechlorination, this process is of major significance in their metabolism and the comparative detoxication of DDT can be conveniently considered here.

In dogs, rabbits, and rats the only excreted metabolic product definitely identified is DDA (*280–283*). Oral doses of DDT are poorly absorbed and much of the dose in rats is excreted unchanged in the feces. Less than 5% is eliminated via the urine, and this is mainly DDA and, possibly, a conjugate of this, though neutral metabolites which give DDA on boiling with acid or alkali are also present (*283*). The main route of excretion of absorbed DDT in vertebrates is the bile, and 65% of an intravenous dose of C^{14}-DDT can be found as radioactivity in the bile (*280, 284*). Only a trace of DDT itself is excreted in the bile, and again DDA is a major product. Only part of the radioactive products in the bile can be estimated as DDA by the Schechter-Haller process or by absorption spectrum, but this amount can be considerably increased by acid hydrolysis. It has been suggested (*284*) that the conjugated form of DDA in the bile is a glycine conjugate, although the related compound diphenylacetic acid does not form a glycine conjugate (*285*). The excretion pattern in bile is complicated; several radioactive products in addition to DDA have been detected in radioactive chromatograms (*284*).

The dehydrochlorination product DDE can also be detected in vertebrates (*286*), but this is not excreted in more than trace amounts: it accumulates in the body fat. Samples of human fat were all found to contain DDE and DDT, presumably accidentally absorbed (*287*). No DDA or 4,4′-dichlorobenzophenone was found in vertebrate fat after dosing with DDT (*288*). The bromophenyl analog of DDT behaves similarly in vertebrates: an acidic metabolite of this compound analogous to DDA has been detected in urine of hens, rats, and man (*289*).

Methoxychlor, the *p*-methoxyphenyl analog of DDT, is also excreted mainly in the bile in mammals though the metabolites have not been identified; only about 5% of the dose appears in the urine. A strongly acid metabolite, not analogous to DDA, is present in rabbit urine and also a weak acid, perhaps a phenol formed by demethylation (*278, 290, 291*).

The metabolism of DDT in insects has received more attention and the subject is correspondingly more complex. Much of the work has been carried out on resistant houseflies, but it is now clear that considerable species differences exist among the insects and a number of possible metabolites have been proposed or partially identified (Table VI). In most cases no positive identification of the possible metabolites listed in this table is claimed by the authors quoted, but it is clear that DDE is by no means the only possible metabolite of DDT in insects.

The first insect studied was the large milkweed bug, *Oncopeltus fasciatus,* and this has set the pattern for much succeeding work. Injected DDT was rapidly destroyed and only small or negligible amounts of DDE were measurable, most of the dose being converted to substances not responding to the Schechter-Haller test. This has recently been confirmed with C^{14}-DDT (*292*).

In normal and resistant houseflies the formation of DDE is of more importance, and though the identification of this metabolite is often made as a result of color reactions or paper chromatography, the dehydrochlorination process, in flies at any rate, is now firmly established by the separation of the enzyme concerned (*293–295*). The fly DDT-dehydrochlorinase acts only on DDT and close chemical analogs; it requires glutathione or cysteinylglycine as specific cofactors and is not activated by other SH compounds or CN^-. It differs in this respect from the dechlorinating enzyme of rats, studied by Heppel and Porterfield (*275*). The activity of the enzyme correlates satisfactorily with the size of the toxic dose required to kill the various strains of DDT-resistant houseflies (*293*); a careful examination of the analytical recoveries of DDT and DDE in seven resistant strains (*296*) showed that the formation of DDE was the main factor in the low toxicity of DDT in these strains.

It is possible that the water-soluble, non-Schechter-Haller metabolites formed in flies are the products of further metabolism of DDE. In some cases these metabolites may form a large proportion of the excreted radioactivity after dosing with C^{14}-DDT. Thus Terriere and Schonbrod (*29*) using similar doses and times to those used by Perry *et al.* (*296*) found that up to 88% of the dose of DDT was excreted by houseflies as water-soluble products; no great difference was seen between the resistant and normal strain. In their experiments, much more of the dose of C^{14}-DDT was excreted than in those of Perry *et al.* (*296*), and it seems probable that in insects, as in mammals, the nonpolar metabolite DDE accumulates in the fatty tissues. The rapidity with which this is further metabolized to water-soluble metabolites that can be easily excreted, may depend, even in the same strain of fly, on uncontrolled factors such as the amount of fatty tissue present. The identity of these water-soluble materials is not known, but evidence for some conjugated phenolic material was found.

TABLE VI
Metabolites of DDT in Some Insect Species

Species	Technique[a]	Time	DDT	DDE	Unidentified[b]	Tentative identification	Reference
Oncopeltus fasciatus	Col.	1.5 Hr.	+	—	+	—	*(302)*
Oncopeltus fasciatus	C^{14}, chrom.	8 Days	+	5%	+	—	*(292)*
Aedes aegypti	Col.	—	—	10%	—	—	*(303)*
Periplaneta americana	C^{14}, chrom.	—	5%	5%	90%, 5 metabs.	—	*(297)*
Periplaneta americana	C^{14}, chrom.	1 Day	40%	4%	3 metabs.	Dichlorobenzophenone	*(292)*
Periplaneta americana	Chrom.	—	—	+	+	Dichlorbenzhydrol	*(304)*
Leucophaea maderae	C^{14}, chrom.	23 Days	12%	2%	77%, 3 metabs.	—	*(298)*
Pyrausta nubitalis	C^{14}, chrom.	72 Hr.	63%	30%	—	—	*(298)*
Argyrotenia velutinana	Col.	168 Hr.	63%	20%	—	—	*(305)*
Epilachna varivestis	Col.	72 Hr.	3%	23%	74%	—	*(305)*
Melanoplus differentialis	Col.	4 Days	54%	41%	—	—	*(305)*
Melanoplus femur rubrum	Col.	8 Days	34%	59%	—	—	*(305)*
Sarcophaga crassipalpis	Chrom.	—	—	+	+	Dichlorbenzhydrol	*(304)*
Pediculus humanus	C^{14}, col.	24 Hr.	—	Nil	82%	*p*-Chlorobenzoic acid	*(300)*
Musca domestica	C^{14}, col.	—	—	+	—	—	*(296)*
Musca domestica	C^{14}	8 Days	—	—	58% Water sol.	Phenol conjugate	*(29)*
Musca domestica	Col.	—	—	+	+	—	*(306)*
Musca domestica	C^{14}, chrom.	24 Hr.	6%	60%	34%	—	*(292)*
Bombyx mori	C^{14}, chrom.	72 Hr.	88%	12%	—	—	*(292)*
Nymphalis antiopa	C^{14}, chrom.	72 Hr.	69%	2%	29%	—	*(292)*
Malacosoma americana	C^{14}, chrom.	19 Hr.	15%	66%	19%	—	*(292)*
Plodia interpunctata	C^{14}, chrom.	144 Hr.	26%	26%	56%	—	*(292)*
Ornithodorus coriaceous	C^{14}, chrom.	96 Hr.	4%	Nil	96%	—	*(292)*
Drosophila melanogaster	C^{14}, chrom.	—	—	+	+	1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethanol (Kelthane)	*(301)*
Anthonomus grandis	Col.	2 Days	—	Trace	40%	—	*(307)*

[a] C^{14}, using radioactive DDT; col., based on Schechter-Haller color reaction; chrom., paper or adsorption column separations.

[b] Metabs. = metabolites.

In other insects dehydrochlorination is not always the main metabolic route. In the cockroaches *Periplaneta* and *Leucophaea* (*297*), DDE is a minor metabolite and up to five other products make up the bulk of the excreted material. Lindquist and Dahm (*298*) consider that, in the Madeira roach, two of these are direct metabolites of DDT and a minor one may be derived from DDE. One or more of these metabolites may be a carbohydrate conjugate of some kind (*299*).

In Korean lice, DDT is destroyed rapidly but no DDE is formed. Enzyme assays also show the absence of DDT dehydrochlorinase. The metabolite was acidic; *p*-chlorobenzoic acid was the only substance considered which had properties similar to the metabolite, but no identification was attempted (*300*).

The best-characterized metabolite from an insect which does not form DDE is that formed in *Drosophila melanogaster*. Resistance to DDT in this species is controlled by a dominant gene which has been located in the second chromosome and is associated with the formation of 1,1-bis-*p*-chlorophenyl-2,2,2-trichloroethanol. The metabolite was identified by its identity of behavior with this compound in a number of partition and adsorption chromatography systems; it had the same absorption spectrum and color reactions. This compound was already known as the acaricide Kelthane, and it is remarkable that this detoxication product (at least six times less toxic than DDT to the flies) of an insect is an effective toxicant for mites. It is also significant that Kelthane is easily degraded by alkali to dichlorobenzophenone, which has been suspected as a possible metabolite of DDT in several other insects (Table VI), and it is also possible that the colorimetrically measured "DDE" in some cases may be either Kelthane or an artifact formed from it (*301*).

III. Reaction Rates and Species Differences

Reaction rates of a single detoxication reaction in cold-blooded animals may be greatly affected by environmental temperature changes, which may cause apparent species differences in metabolism. Frogs at 25° are very tolerant of colchicine, which is highly toxic in mammals. At 37°, however, the frog is killed as readily as a warm-blooded animal, and this has been attributed to the more rapid conversion of colchicine to a toxic oxidation product at the higher temperature (*308*).

Insects are killed more easily at 15° than at 35° by pyrethrins, probably because the balance between inhibition of essential functions and inactivation favors inactivation at the higher temperature. In agreement with this, it is found that the esterase inhibitor piperonyl butoxide increases susceptibility at higher temperatures (*309*).

DDT is more effective, by injection or topical application, at 16° than at 30° as the rate of inactivation by dehydrochlorination increases at the higher temperatures (*310*).

In warm-blooded animals and in cases where several detoxication mechanisms may be applied to a compound, the differences in excreted metabolites can often be referred to different rates of reaction of the various possible metabolic routes. Since the exploitation of these differences in alternative routes is useful in the design of selectively active compounds, it would be desirable to have some quantitative kinetic data for the various reactions. It might then be possible to forecast metabolic pathways of a new compound.

Studies on several vertebrate species suggest that this may be difficult to achieve in practice. Dogs, for example, conjugate benzoic acid mainly with glucuronic acid, and rabbits use mainly glycine. It is pot possible to predict from this the fate of other aromatic acids; *m*-aminobenzoic acid, for instance, is conjugated predominantly with glycine in both species (*311*). Recent kinetic studies on these conjugations and also other studies on sulfate conjugation and oxidations (*144, 311, 312*) suggest that major factors governing the choice of two alternative metabolic routes are the first- and zero-order rate constants of the two reactions.

The former, in conjugation reactions, may not be subject to serious species variations, but the zero-order rate constant depends not only on the mobilization rate of the glycine, glucuronic acid, etc., which is different in different species, but also on the compound metabolized. The compound itself may affect the zero-order constant either by virtue of its tissue level (*311*), which depends on the dose, or by its effect on the maximum velocity of the enzymatic reaction (*312*).

If a zero-order reaction rate could be established for the detoxication of a particular compound in a given species, the extrapolation of the result to related unexamined compounds might be possible if only a single detoxication mechanism were involved. It is becoming increasingly apparent, however, that the detoxication mechanisms often involve several enzymes with different specificities (e.g., demethylation, nitroreduction, hydroxylconjugation, and probably hydroxylation). Forecasting the extent of a detoxication reaction in one species from results in another, where the relative amounts of the various enzymes differ, would be even more difficult.

A qualitative approach is, however, useful in suggesting possible structures for investigation and promising results have been obtained in designing selectively active organophosphate molecules. Compounds of this class form very suitable design subjects as their toxic activity usually depends on a metabolic activation and the balance between this and various inactivation reactions may be tipped by quite small species differences in the various reaction rates. Much greater differences in biological activity can result from the balance of two reactions than from the often small species differences in rates of a single inactivation process.

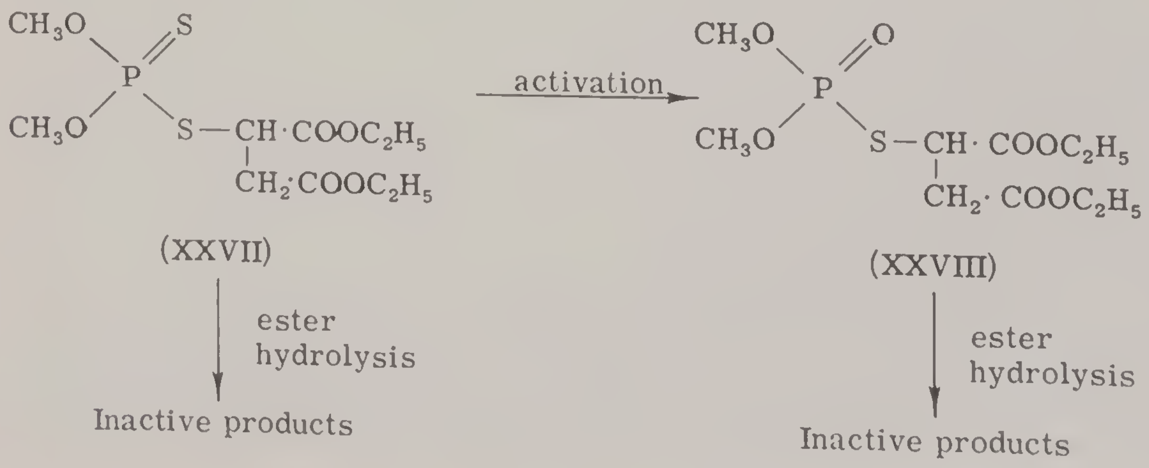
The toxicity of malaoxon (XXVII) is similar for insects and mammals (Table VII), but malathion (XXVIII) may be much more toxic to insects than to mammals. The toxic action of malathion is exerted mainly through its oxidation product malaoxon, and this reaction occurs somewhat more readily in insects (*313*). Deactivation takes place simultaneously by esterhydrolysis and phosphatase action, and this occurs more rapidly in the mammals. The rates of activation and deactivation, as expressed by the amount of oxidized metabolites or hydrolysis products present after half an hour are not very different in mice and insects, but in insects the balance favors the accumulation of the lethal metabolite malaoxon.

TABLE VII

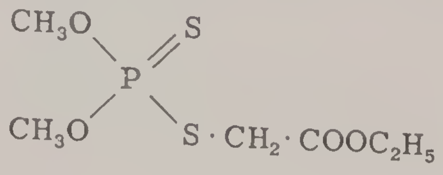
TOXICITY AND METABOLISM OF MALATHION[a]

Organism	Malathion (LD_{50})	Malaoxon (μg./gm.)	Dose (μg./gm.)	Per cent of water-soluble metabolites as: Oxidation products	Carboxyesterase products
Cockroach	16	1.5	4	11	50
			40	2	43
Housefly	30	15	15	7	27
			150	23	24
Mouse	1590	75	30	2.5	68

[a] Condensed from Krueger and O'Brien (*313*).



Synthesis of other structures to take advantage of the apparently greater hydrolysis rate in mammals was undertaken (*314*), e.g., acethion (XXIX), and these compounds were less toxic to mice than to flies.



(XXIX)

Unexpectedly, acethion was not toxic in cockroaches as these insects were able to hydrolyze the ester groups as rapidly as mice. Other examples using this principle of a center for activation and a center for deactivation in the molecule are quoted by O'Brien (*314*), and in contrast to the older, universally toxic organophosphates, compounds are now known with such favorable selective action that they may be used as systemic insecticides in cattle (*315*).

IV. Developmental Aspects of Detoxication

This aspect of the subject has received little attention in the past and it is unwise to draw any general conclusions in spite of the temptation to look for a relation between the time of appearance of a detoxication mechanism in the embryo and its phylogenetic distribution.

The ubiquitous rhodanese system is present in detectable amounts in fertile hen's eggs after 4 days' incubation (*252*) and is present from an early stage in fetal rat liver (*250, 316*). The ethereal sulfate synthesis is also present in eggs at an early stage. The detection of ethereal sulfate in human amniotic fluid at birth is one of the earliest references to this class of compound (*317*). The ethereal sulfate concentration of fetal blood is also said to be significantly higher than of that of the mother (*318*).

While these blood analyses do not necessarily indicate a fetal synthesis of ethereal sulfate, this view is supported by the direct determination of the synthesis in embryonic chick and rat liver and intestine (*319*). The activity of the embryo chick liver was about ten times that of the newly hatched bird, but the embryonic enzyme system was much more heat labile than that of the adult, and it is possible that different enzymes are active in embryo and adult.

In earlier work, Takahashi (*320*) had shown that the sulfate-esterifying system was active in the 9-day chick embryo when no inorganic sulfate could be found. Takahashi's experimental figures are somewhat scattered, but they show a clear difference between the total and inorganic SO_4^{2-}. The absence of any nonesterified SO_4^{2-} in fertile eggs at this stage of growth was noted also by Needham (*321*).

The early appearance of this classic detoxication mechanism in embryonic tissue has occasioned some surprise since it had been commonly assumed that its function was the inactivation of phenols formed by the intestinal microorganisms. Since this is not necessary in the embryo it was thought possible that the ethereal sulfates were derived from phenolic by-products of amino acid metabolism. Recent reports, however, seem to show that the hydroxylation of aromatic amino acids, which might give the presumed phenolic by-products, is a mechanism which does not develop in rats, pigs, rabbits, and children until shortly before birth (*322*). Another possibility, considered by Lindahl in his study of the sea urchin egg, is that an ethereal sulfate may have some sort of hormonal function and that the lack of this may be associated with the abnormal development of these eggs in SO_4^{2-}-free sea water (*323, 324*). The presence of ethereal sulfate has not been demonstrated in the undivided sea urchin egg, but the presence of arylsulfatase in the eggs (*325*) may indicate the presence of a suitable substrate.

Except for the presence of the sulfate conjugation in vertebrates and arthropods, it has only been found in a special group of mollusks and in one mold. Many more data are necessary before it can be decided whether, as the embryological results seem to suggest, it is a primitive biochemical characteristic or whether its occurrence in the highly evolved organisms is another example of convergence in evolution.

In contrast to the ethereal sulfate synthesis, glucuronide formation in mammals only appears in fetal tissue shortly before birth. A similar observation is that the capacity for conjugating phenols, either as sulfate or glucuronide, only appears in frogs and toads after metamorphosis (*100*). In the case of the mammalian fetus both the UDPG dehydrogenase and UDPGA-phenol transferase appear at about the same time (*326, 327*), and in guinea pigs, mice, and man the full adult activities of the enzymes are not reached for several weeks after birth.

While the embryo does not seem able to conjugate carboxylic acids with glucuronic acid, there is some evidence that the conjugation with glycine or ornithine is present from about half way through development.

Fetal amniotic and allantoic fluid from sheep and cows contains hippuric acid, and in sheep up to 20% of the nitrogen content of allantoic fluid may be present in this form, but the concentration falls considerably toward term (*328*). Despite the relatively high concentrations reported, it is not certain that the hippuric acid is formed by the fetus. In developing eggs, however, direct demonstrations of the ornithuric acid synthesis has been possible after injection of benzoic acid. Takahashi (*329*) reported that no glycine or ornithine conjugates could be isolated from hen's eggs injected with benzoic acid at 9 days' incubation, but ornithuric acid was extracted from the eggs after 14 or 18 days. It appears therefore that the ornithuric acid conjugation arises rather later in the hen than does the sulfate conjugation. It is also significant that at about 10–12 days the nitrogen metabolism in the developing embryo becomes primarily uricotelic and that it is only those species with a uricotelic nitrogen metabolism which are capable of the ornithuric acid detoxication.

A number of detoxication processes in mammals are associated with the liver microsomes. In addition to the glucuronic acid detoxication already mentioned, the liver microsomes carry out a number of oxidations dependent on the presence of TPNH and O_2 and have a nitroreductase activity. These reactions are not catalyzed by the liver microsomes from fish, amphibia, reptiles, and birds, though the reptiles and birds and some amphibia have alternative detoxication systems. It is of considerable interest, therefore, to find that the liver microsomes of mammals acquire their characteristic complex of detoxication enzymes only at about the time of birth and that these reach their full adult activity some weeks later (*330*) (Table VIII).

TABLE VIII
RELATIVE ACTIVITIES OF DETOXICATION MECHANISMS IN INFANT AND ADULT RABBIT LIVER HOMOGENATES[a]

Activity	2 Weeks	3 Weeks	Adult
Hexobarbital side-chain oxidation	0.96	2.8	8.4
Aminopyrine demethylation	0.15	0.3	3.25
l-Amphetamine deamination	1.3	1.9	8.8
Acetanilide hydroxylation	1.0	1.9	4.5
Chlorpromazine *S*-oxidation	11.4	31.5	32.3
p-Nitrobenzoic acid reduction	1.72	5.16	9.9

[a] After Fouts and Adamson (*330*).

The nonmicrosomal system in toads which oxidizes hexobarbital and aminopyrine, and which is absent in fishes, similarly appears at metamorphosis (*67*, *100*).

Similar detailed results are not available for developing avian eggs, but it is probable that the oxidation systems of the egg are less effective than in the adult. Pentobarbital and chlorpromazine, which are extensively metabolized in adult mammals, are excreted unchanged in the allantoic or amniotic fluid of developing eggs (*331*). Quinine on the other hand, which is oxidized by an enzyme separate from the microsomal group in mammals, is destroyed in the 10–16-day embryo more rapidly than in the adult bird (*332*).

Conjugation, presumably acetylation, of aromatic amino groups of sulfonamides occurs extensively in developing hen's eggs and up to 75% of a dose of sulfacetimide may be present in this form after 48 hours (*333, 334*).

V. Detoxication and the Adoption of a Terrestrial Habitat

To a great extent the known species differences in detoxication mechanisms are quantitative in nature; qualitative differences are found only among the peptide and sugar conjugations. This may be an indication that many of the reactions, e.g., oxidation, reduction, hydrolysis, thiocyanate formation, and possibly acetylation, are carried out by normal intermediary metabolic systems in many species. The conjugations with amino acids, sugars, and sulfate have a less obvious connection with normal metabolism and are usually achieved by specific enzymes, but even these use "activated" forms of sulfate or sugar which are synthesized for other, normal, processes in the cell. It is perhaps possible that these conjugations have evolved to assist in the elimination of toxic by-products of metabolism and unnecessary foreign compounds inevitably taken in with the food. It is significant that while the conjugates with sugars and sulfate are invariably nontoxic, oxidation, hydrolysis, and so on may yield toxic or nontoxic metabolites in a quite fortuitous fashion.

The view that detoxication mechanisms are special functions rather than accidental involvements of normal metabolism has recently been supported by Brodie *et al.* (*67*). The higher vertebrates have in their liver microsomes a set of oxidative mechanisms which metabolize a variety of lipid-soluble foreign compounds. Similar systems are absent from several lower vertebrates and aquatic species, and it is suggested that the microsomal complex is a specially evolved detoxication system. The lower vertebrates do nevertheless carry out many detoxication reactions, but in different sites and probably by different mechanisms than those of higher vertebrates.

In discussing these results Brodie *et al.* (*67*) suggested that the development of the microsomal enzymes and detoxication mechanisms was associated with the colonization of dry land and the development of the kidney. The ideal detoxication product for an organism with a hypertonic urine is water soluble and unable to pass through lipoid membranes, so that after glomerular filtration it is left in the kidney tubule as the water is resorbed. This is usually better fulfilled by the sugar or sulfate conjugates than by the initial products of oxidation or hydrolysis or by the original, usually fat-soluble, organic compounds.

It was felt that the ample water supply of fishes and aquatic stages, such as tadpoles, made detoxication mechanisms unnecessary in these organisms, but examples are known of aquatic species (e.g., mosquito larvae, adult water beetles, whales, dogfish) that possess conjugation mechanisms.

Water conservation and the elimination of unwanted products is achieved by similar processes in all terrestrial animals (*335*). The essentials of an excretion system are filtration of the blood in a glomerulus or Malpighian tube followed by water resorption in a kidney tubule or rectal gland, but in vertebrate kidneys the acidic detoxication products, glucuronides, and ethereal sulfates are also actively secreted by the tubules (*336*). This is an additional process tending to concentrate the vertebrate detoxication products in the urine. No corresponding structure or process appears to be present in insects, and the characteristic conjugate is a glucoside which fulfills the main criterion for a detoxication product, namely, a high water:fat partition ratio. It must be remembered, however, that the highly acidic ethereal sulfates are also found in both insects and vertebrates.

The freshwater fishes, in which the glucuronide conjugation has been studied, all have an abundant water supply and a copious hypotonic urine. In these circumstances the glucuronides or sulfates would have no great advantage over the water-soluble phenols as excretion products and it is found that these species do not form these conjugates *in vivo.* Fish livers do, however, contain a conjugating system (*116*), and two marine species, *Oncorhynchus tschawytsha* (*337*) and *Gadus callarias,* are known to synthesize UDPGA. Since these marine species, like terrestrial animals, have a water conservation problem it would be of some interest to see whether a mechanism for excreting glucuronides has developed as with the terrestrial organisms.

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AUTHOR INDEX

Numbers in parentheses are reference numbers and indicate that an author's work is referred to although his name is not cited in the text.

Numbers in italic show the page on which the complete reference is listed.

A

Aaes-Jørgenson, A., 193(207), *219*
Abdelkader, M. M., 425(183), 426(183), *453*
Abderhalden, E., 388(270), *401*
Abelson, P. H., 75(4), *107*
Ablondi, F. B., 105(316), *117*
Abraham, M., 107(339), *117*
Abramowicz, A. A., 264(135–137), *294*
Abramowicz, R. K., 264(137), *294*
Abramsky, *see* Avron
Acher, R., 304(26), 307(29, 31, 32, 33, 36, 37), 308(38, 39, 41), 309(43), 310(31, 32, 55), 311(31, 32), 312(37, 38, 39, 60), 313(37), 314(65), *341, 342*
Adam, A., 104(306), 105(306), *116*
Adams, E., 96(242), *114*
Adamson, R. H., 446(330), *456*
Adamsons, K., Jr., 302(3), 303(3), 307(34), *340, 341*
Adelberg, E. A., 95(236), 105(236), *114*
Adolph, E. F., 147(90), *216*
Afridi, M. M. R. K., 90(190), 91(190), *113*
Agrell, I., 224(4, 5), 225(4), 229(5), 231(5), 232(5, 80), *241, 243*
Ahmed, M. K., 410(55), *450*
Aisenberg, A. C., 80(57), *109*
Akabori, S., 132(44), *215*
Akazawa, T., 38(94), *69*
Akesson, B., 260(92), *293*
Albert, A., 404(4), *448*
Alexander, F., 447(334), *457*
Alexandrowicz, J. S., 262(119), *293*
Al-Hussaini, A. H., 376(187), *398*
Allen, M. B., 88(146), *112*, 206(235), 207(235), *220*
Allen, S. H. G., 28(77), *69*
Allison, J. B., 261(106), *293*
Allman, D., 85(134), *111*
Almquist, H. J., 149(95), *216*
Amaha, M., 15(42), *68*
Amanieu, M., 227(38), *241*, 386(258), *400*
Ames, B. N., 76(14), 77(20), 96(20), 97(20), 98(20), *108*
Amiard, G., 310(52), *341*
Anagnostopoulos, C., 96(255), *115*
Anders, E., 126(32), 135(53), *214, 215*
Andersen, R. N., 319(87, 88), *342, 343*
Anderson, E. P., 166(135), *217*
Anderson, I. G., 431(228), *454*
Anderson, J. M., 228(50), *242*
Anderson, M. L., 14(38), 22(67), 23(67), 24(38, 71), 25(38), 26(38), 28(71), *68, 69*
Anderson, R. C., 424(178), *453*
Andrae, W., 417(133), 418(103), *451, 452*
Annison, E. F., 362(118a), 363(118a). 365(118a), 366(118a), *396*
App, A. A., 90(192), *113*
Arima, K., 84(97), 86(97), *110*
Armstrong, E. F., 418(101), *451*
Arnon, D. I., 90(188), *113*
Arnstein, H. R. V., 430(218), *454*
Arrhenius, S., 128(40), *214*
Arvy, L., 258(61), *292*
Ash, R. W., 364(150a), *397*
Ashcraft, D. W., 370(162), *397*
Ashkenazi, I., 104(305, 306), 105(306), *116*
Ashmore, J., 107(348), *118*
Ashton, D. M., 78(21), *108*
Astwood, E. B., 319(83), 320(83), *342*
Asyamolova, I. A., 356(86), 357(87), *394*
Auerbach, V. H., 92(198), 103(288, 289), 107(198), *113*, *116*, 357(86a), *394*

Avel, M., 260(83), *292*
Avron, M., 80(55), *109*
Axelrod, J., 405(12), 410(61), 423 (161), 426(189), *449*, *450*, *452*, *453*

B

Baars, J. K., 137(59), *215*
Babkin, B. P., 348(10), 357(10), 374 (181, 182), 376(182), *392*, *398*
Bach, J. A., 16(44), 18(44), *68*
Backström, S., 42(121), *70*
Bacq, Z. M., 248(5), 249(5, 6, 10), 250 (5, 6), 251(5), 254(5, 37, 48), 283(264), *291*, *292*, *297*
Baer, J. E., 412(77), *450*
Bain, J. A., 78(25), 102(25), *108*
Bain, W. A., 254(40), *291*
Baker, C., 438(287), *455*
Baker, F. A., 102(283), *115*
Baldini, J. T., 317(72), *342*
Baldwin, B. C., 414(94), 421(150, 151), 428(206), 429(206), *451*, *452*, *453*
Baldwin, E., 139(67), *215*, 315(67), *342*, 348(2), 350(2), 356(2), *392*
Balesdent-Marquet, M. L., 271(204), *296*
Balfour, W., 251(18), *291*
Ballesteros, R. B., 434(255), *454*
Ballien, M., 352(51, 52, 53), 353(51, 52, 53), 373(51), *393*
Balls, A. K., 383(235), *399*
Band, R. N., 89(153), *112*
Banerjee, G., 107(341), *117*
Banerjee, S., 430(217), *454*
Banks, E. M., 359(108), *395*
Bann, J. M., 411(71), *450*
Bárány, E., 349(23), *392*
Barber, M., 436(269), *455*
Barbier, M., 288(293a, 297), *298*
Barcroft, J., 362(122, 123, 125), *396*
Barker, D. E., 352(55), *393*
Barker, H. A., 201(227, 228), 202(229), *220*
Barker, R. J., 442(310), *456*
Barnafi, L., 319(93), 322(107), 324 (108), *343*
Barner, H. D., 18(52), *68*
Barnes, M., 433(239), *454*
Barnes, M. R., 52(155), *71*
Barnett, A. J. G., 365(150f), *397*
Barnett, H. L., 122(8), 139(8), *214*
Barnett, J. A., 99(263), *115*
Barrett, J. T., 85(126), 93(126), *111*
Barrington, E. J. W., 283(261), 284 (261), 285(273), *297*, 356(86d), 375(190), 376(190), 377(190), 381 (228), *394*, *398*, *399*, *401*
Barrows, C., 444(319), *456*
Barry, B. A., 353(60e), *393*
Barsa, M. C., 224(12–16), 226(32), *241*
Barth, L. G., 56(162), *71*
Barth, L. J., 56(162), *71*
Bartlett, M. F., 312(58), *342*
Bartley, W. C., 423(157), *452*
Bartollota, A. J., 228(52), *242*
Bartos, J., 310(52), *341*
Batt, R. D., 84(112), 87(112), *111*
Bauer, G. C. H., 169(144a), *217*
Bazinet, M., 287(286a), *298*
Beatty, I. M., 160(123), *217*
Beavers, H., 412(80), *450*
Becker, E., 272(208, 210), *296*
Beckett, A. H., 413(84), *450*
Beckmann, R., 286(277, 278), *298*
Beckwith, J., 78(36), *108*
Beermann, W., 237(107), *243*
Beerstecher, E., Jr., 122(12), 126(30), 127(34), 128(34), 136(34), 139 (12), 146(34), 157(117, 118), 165 (133), 175(34), 180(171), 186 (34), 188(192, 193), 189(192), 195(133), 197(133), 207(240), *214*, *217*, *218*, *219*, *220*
Beevers, H., 29(80), 35(90), 36(93), 37(93), 38(93, 94), *69*
Begemann, H., 354(63), 355(63), *394*
Begg, R. W., 137(58), 174(58), 177 (58), *215*
Bell, P. H., 320(95), 325(114), 327 (114), 330(114), *343*
Ben Abdeljlil, A., 357(*see* footnote), *401*
Benati, O., 248(4), *291*
Bendall, D. S., 80(56), *109*
Benfey, B. G., 319(91), *343*
Ben-Gershom, E., 79(48), *109*
Bennett, S. H., 410(56), 411(56), *450*
Benson, E. R., 254(49), *292*

Bentley, O. G., 182(173), *218*
Bentley, P. J., 303(15), *340*
Benzer, S., 82(70), *110*
Berde, B., 302(4), 305(4), *340*
Berg, O., 284(266), *297*
Berg, W. E., 40(106), *70*
Bergmann, W., 208(246, 247, 248), *220*
Bergquist, A., 77(19), *108*
Bergstrand, A., 44(129), *70*
Bergström, S., 78(28), *108*
Berkeley, C., 384(239g), *400*
Berkman, J. I., 202(232), *220*
Berliner, D. L., 92(211), 93(211), *113*
Berliner, M. L., 92(211), 93(211), *113*
Bernal, J. D., 132(46), *215*
Bernheim, F., 84(110), *111*
Beroza, M., 122(21), *214*, 286(283), 287 (284a, 286b), *298*
Bersin, T., 356(86c), *394*
Bertran, E. C., 434(247, 251–257), 444 (251), *454*, *455*
Best, C. H., 348(8), 357(8), *392*
Bettini, S., 425(176), 433(243), *453*, *454*
Beutler, R., 380(221), *399*
Bevelander, G., 168(141), *217*
Bezuk, A. B. L., 254(41), *291*
Biale, J. B., 80(55), *109*
Biedermann, W., 378(215), 385(245), *399*, *400*
Biesele, J. J., 210(255), *220*
Bieter, R. N., 424(169), *452*
Bigger, J. H., 411(74), *450*
Bigley, D., 81(66), 84(66), 96(66), *109*
Bird, O. D., 200(222), *219*
Bishai, F., 230(60), *242*
Bishop, D. W., 80(64), *109*
Bissell, H. D., 364(143), *396*
Black, E. C., 377(197), *398*
Black, E. D., 287(286a), *298*
Black, R. E., 39(103), *70*
Blackburn, T. H., 362(127), 363(127), 364(150d), *396*, *397*
Blackith, R. E., 435(263), *455*
Blackwelder, E., 127(36), *214*
Blair, D. G. R., 78(34), *108*
Blatt, W. F., 105(320), *117*
Blaxter, K. L., 365(150h), *397*
Blewett, M., 192(202), 193(202), *219*
Bloch, K., 79(38), *108*, 423(160), *452*
Bloom, B., 24(73), 25(73), 28(73), *69*
Blum, M. S., 440(307), 441(309), *456*
Blumenthal, H. J., 15(43), *68*, 96(254), 99(254), *115*
Blunck, H., 388(276), *401*
Boaz, N. F., 317(71), *342*
Bobin, G., 258(64, 67), *292*
Boccacci, M., 425(176), 433(243), *453*, *454*
Boch, R., 288(294a,b), *298*
Bodanszky, M., 310(53), *341*
Bodenstein, D., 224(17), *241*, 279(246), *297*
Bodine, J. H., 225(30a), *241*
Boell, E. J., 52(153), 53(157), 59(174), *71*, 225(30a), *241*
Boezi, J. A., 85(125), 105(297), *111*
Bogoraze, D., 271(194, 195), *295*
Boissonnas, R. A., 310(50), 312(59), 321(102, 103, 104), 322(103, 104), 327(119, 120), 328(102, 125), 330 (125), *341*, *342*, *343*, *344*
Bojarski, T. B., 104(296, 297), 105 (297), 106(296), *116*
Bold, H. C., 88(147), *112*
Bolton, E. T., 75(4), *107*
Bone, A. D., 78(30), 79(30), *108*
Bonner, D. M., 74(2), 89(175), 90 (175), *107*, *112*
Bonner, J., 122(9), 139(9), *214*
Bonner, J. T., 45(134), *70*, 122(22), *214*
Bonser, G. M., 407(32), *449*
Booth, J., 408(41), 433(242), *449*, *454*
Borei, H., 38(97), 39(97), 50(148), *69*, *71*
Borgström, B., 351(38a), 359(107), 384 (38a), *393*, *395*
Borkenhagen, L. F., 79(38b), *109*
Borman, A., 337(167), *345*
Bouchilloux, S., 422(141), *452*
Boucly-Urison, M., 424(180), *453*
Bounhiol, J. J., 277(224), 278(224), *296*
Bourgeois, S., 96(243), *114*
Bourne, G. H., 378(205), *398*
Bowen, V. T., 182(174), *218*
Bowery, T. G., 412(79), *450*
Bowie, D. J., 374(182), 376(182), *398*

Boyer, P. D., 195(211), *219*, 254(46), *292*, 356(86b), *394*
Boyland, E., 407(33), 408(41), 411(68), 432(236), 433(242), *449*, *450*, *454*
Boyle, F. P., 406(23), *449*
Boynton, L. C., 147(88), *216*
Brachet, J., 53(159), 57(159), *71*
Bradbury, F. R., 433(246), 437(277), *454*, *455*
Bradley, S. G., 25(74), 26(74), *69*, 90(180), *113*, 163(129), *217*
Braunstein, A. E., 128(39), 131(39), *214*
Brawerman, G., 88(141), *111*
Bray, H. G., 413(93), 422(144), 423(163), 433(241), 442(144, 312), *451*, *452*, *454*, *456*
Bray, R., 153(104), *216*
Brecher, K., 233(86), *243*
Breed, R. S., 143(75), *215*
Bremer, J., 431(227), *454*
Bresnick, E., 78(24), *108*
Breusch, F. L., 351(38), *393*
Bricteux-Grégoire, S., 227(42, 43), 228(43), *242*
Bridges, P. M., 435(264), *455*
Brink, G., 370(160), *397*
Britten, R. J., 75(4), *107*
Brock, T. D., 82(74), *110*
Brockmann, H., 154(106), *216*
Brodie, A. F., 413(86), *451*
Brodie, B. B., 405(11, 12), 409(51), 410(61, 62), 411(66, 67), 412(77, 78), 413(89), 414(100), 420(123, 139), 421(139), 423(161), 445(100), 446(67, 100), 447(67), *449*, *450*, *451*, *452*
Brodie, T. G., 349(21, 22), *392*
Bronstein, S. B., 107(341), *117*
Bronsweig, R. D., 22(66), 23(66), *69*
Brooks, M. A., 151(99), *216*
Brown, A., 99(270), *115*
Brown, A. K., 445(327), *456*
Brown, A. W. A., 434(258), 440(303), *455*, *456*
Brown, B., 76(8, 9), 96(9), *108*
Brown, B. R., 415(129), *452*
Brown, F. A., Jr., 169(143), *217*, 263(129), 264(129), *294*, 348(13), 356(13), 357(13), 378(13), *392*
Brown, G. M., 188(189), 189(189), *219*
Brown, G. V., 107(333), *117*
Brown, G. W., Jr., 61(178), 62(178), *71*
Brown, H., 336(160), *344*
Brown, J. S., 88(146), *112*
Brown, M., 364(150d), *397*
Brown, M. E., 122(15), 139(15), *214*
Brown, R. A., 333(142), *344*
Brown, W. C. B., 362(124), *396*
Brown, W. R., 61(178), 62(178), *71*
Brown-Grant, K., 177(161), *218*
Brunet, P. C. J., 415(126), *451*
Bucher, N. L. R., 101(280), *115*
Buchner, P., 389(280, 281), *401*
Buchs, S., 357(89–90c), 358(89), 359(89–90c), *394*, *395*
Bücher, T., 187(185), 191(185), *218*, 230(60, 61), *242*
Bücker, H., 86(119), *111*
Bückmann, D., 233(87–90), *243*, 275(217, 218), 276(218), *296*
Buckner, A. J., 411(72), 437(274), 440(300), 441(300), *450*, *455*, *456*
Budnick, L. E., 107(350), *118*
Bueding, E., 80(65), 81(65), *109*, 413(87), *451*
Buettner-Janusch, V., 319(86), 320(100), 321(86), 324(86, 109), 325(100), *342*, *343*
Bunge, G., 429(211), 430(211), *453*
Burck, C., 378(217), 379(217), *399*
Burdette, W. J., 266(154), 274(213a), *294*, *296*
Burke, A. W., Jr., 275(222), 277(222), *296*
Burke, J. C., 424(167), *452*
Burkhalter, A., 92(216), 94(216), *114*
Burn, J. H., 303(10), *340*
Burns, E. C., 438(280), *455*
Burst, M., 209(250), *220*
Bushey, M. S., 352(55), *393*
Busnel, R. G., 195(213), *219*
Butcher, E. O., 283(260), *297*
Butenandt, A., 233(91, 92), 235(102), *243*, 272(206), 286(277, 278, 279), 287(287, 289), 288(301), *296*, *298*

Butler, C. G., 288(295), *298*
Butts, J. S., 418(104), 440(299), 441(299), *451*, *456*
Byerrum, R. V., 158(121), *217*
Bykov, K. M., 348(7), *392*
Byrde, R. J. W., 405(14, 15), 409(15, 43), 435(265), *449*, *455*
Byrne, W. L., 79(38a), *108*

C

Cacciola, A. R., 320(95), *343*
Cain, R. B., 409(46), 413(46), *449*
Calaby, J. H., 367(153), *397*
Callow, R. K., 288(295, 296), *298*
Calvery, H. O., 438(283), *455*
Cameron, A. T., 348(11), *392*
Cameron, M. L., 269(180), *295*
Campbell, P. N., 349(24), *392*
Canellakis, E. S., 78(26), *108*
Canellakis, Z. N., 78(26), *108*
Cannon, F. E., 52(154), *71*
Cannon, W. B., 356(80), *394*
Canonica, L., 407(40), *449*
Cantino, E. C., 24(72), *69*, 107(329), *117*
Caputto, R., 357(94, 94a), 358(94, 94a), *395*
Carayon-Gentil, M., 249(9), 250(9), *291*
Cardini, C. E., 416(121), 419(110, 121), *451*
Cardot, P., 139(66), *215*
Carlisle, D. B., 260(93), 263(122, 123, 131), 264(133), 270(190, 190b), 283(265), *293*, *294*, *295*, *297*
Carlson, S. P., 263(126), 264(126), *293*
Carlsson, A., 169(144a), *217*
Carr, C. W., 352(55), *393*
Cartwright, N. J., 409(46), 413(46), *449*
Casanova, R., 259(76), *292*
Casida, J. E., 410(53, 54, 55), 411(53, 54), 429(213), 430(213), 435(53, 54), *450*, *454*
Castellani, O., 166(136), *217*
Catel, W., 356(81), *394*
Cavill, G. W. K., 287(290), *298*
Cazal, P., 271(194, 195), *295*
Ceska, M., 51(150, 151), *71*, 104(307), 105(307, 322), *116*, *117*
Chaikoff, I. L., 107(340), *117*
Chaisson, A. F., 374(181), *398*
Chaix, P., 10(20), *68*
Challenger, F., 426(191, 192), *453*
Chalmers, M. I., 362(126), 363(126), 365(126), *396*
Chaloupka, J., 14(33), *68*
Chamberlain, R. W., 435(261), *455*
Chang, S. C., 440(299), 441(299), *456*
Chang, V. M., 448(337), *457*
Chantrenne, H., 89(177, 178), 90(178), *113*, 430(219), *454*
Chapman, G. B., 262(118), 263(118), *293*
Charniaux-Cotton, H., 271(202, 205), *296*
Chauvet, J., 307(31, 32, 33, 36, 37), 308(38, 39, 41), 310(31, 32, 55), 311(31, 32), 312(37, 38, 39, 60), 313(37), 314(65), *341*, *342*
Chauvet, M. T., 307(33), 312(39, 60), *341*, *342*
Chauvet-Lenci, M. T., 308(41), *341*
Chefurka, W., 224(28), 225(28), 228(44), *241*, *242*
Cheldelin, V. H., 153(103), 211(259), *216*, *220*
Chen, K. K., 424(178), *453*
Chen, P. S., 228(47), 229(56), *242*
Chichester, C. O., 152(101), 206(101), *216*
Child, R. G., 320(95), *343*
Chimo, H., 232(82), *243*
Chiquoine, A. D., 45(134), *70*
Christensen, B. E., 440(299), 441(299), *456*
Christensen, J. J., 89(157), *112*
Christman, D. K., 154(107), *216*
Chung, D., 320(99), 325(99), 327(121), 328(121), *343*
Church, B. D., 5(4), 7(11), 11(25), 15(4), 17(4, 11), 18(11), *67*, *68*
Cipera, J. D., 328(124), *344*
Cirillo, V. P., 89(150), *112*
Civen, M., 92(202, 203, 209), 106(202), *113*
Clare, N. T., 409(50, 52), *450*

Clark, F., 128(39), 131(39), *214*
Clark, M. E., 259(77), *292*
Clark, R. B., 258(62, 65, 68), 259(77), *292*
Clarke, P. H., 85(128), *111*
Classon, O. E., 409(49), *450*
Clauser, H., 304(25, 26), *341*
Clayson, D. B., 407(32), *449*
Clayton, G. W., 317(76), *342*
Clayton, R. K., 84(92, 93), 86(93), *110*
Clements, M., 282(257), *297*
Cleveland, L. R., 275(222), 277(222, 223), *296*
Clever, U., 237(108, 109), 238(109a), *243*, 275(215), 276(215), *296*
Clowes, G. H. A., 38(98), 41(109), 47(140), 48(145), 49(145), 50(147), *70*, *71*
Cocito, C., 95(237a), *114*
Cohan, M. S., 90(191), 91(191), 104(191), *113*
Cohen, G. N., 85(122), 96(246), 97(246), *111*, *114*
Cohen, I., 192(203), *219*
Cohen, P. P., 61(178), 62(178, 182, 183), 67(183), *71*, 107(333, 351), *117*, *118*
Cohen, S., 84(103), 86(103), *110*
Cohen, S. S., 18(52), *68*, 107(343), *117*
Cohen-Bazire, G., 83(80), *110*
Cohn, M., 82(69), 83(69, 85, 86), 95(234), 106(69), *110*, *114*
Cohnheim, O., 354(66), *394*
Cole, R. D., 325(117), 333(143), *343*, *344*
Collins, J. F., 81(66), 84(66), 96(66), *109*
Colovos, N. F., 364(144), *396*
Commerford, S. L., 333(140), *344*
Communol, R., 363(129), *396*
Conney, A. H., 92(206), *113*
Constable, B. J., 317(73), *342*
Conway, E. J., 127(37), *214*
Cook, R. P., 101(277), *115*
Cools, A., 366(151), *397*
Coombes, A. I., 207(237), *220*
Coon, J. M., 303(12), 316(12), *340*
Cooper, J. R., 103(291), *116*, 405(11), 410(61), *449*, *450*
Copenhaver, J. H., 102(286), *116*
Coppée, G., 249(10), *291*
Cordier, D., 354(61), *393*
Cori, C. F., 350(28, 29), *393*
Corteggiani, E., 249(8), 250(8), *291*
Cotty, V. F., 224(6), *241*
Cotzias, G. C., 183(176), *218*
Couch, J. R., 372(169), *397*
Coursaget, J., 92(197), 94(197), *113*
Courtois, C., 89(178), 90(178), *113*
Covelli, I., 285(271), *297*
Cowie, D. B., 75(4), *107*
Cox, R. P., 92(213a), 94(213a), 103(213a, 287a), 107(349), *114*, *116*, *118*
Craig, L. C., 308(42), 312(42), 333(141, 146), 336(159), *341*, *344*, 412(77), *450*
Crane, C., 409(45), *449*
Crane, R. K., 47(142), 48(142), *70*, 78(31, 32), 79(31, 32), *108*, 351(33a), 353(60a), *393*
Cravens, W. W., 372(168), *397*
Creaser, E. H., 19(54), *68*, 84(102), 86(102), *110*
Crebolder, A. J. M., 357(98), *395*
Creevy, D. C., 90(180), *113*
Crepy, D., 308(39), 312(39), *341*
Crombach, J. J. M. L., 374(178), *398*
Crooks, P. G., 15(40), *68*
Cross, B. A., 303(9), *340*
Crowdle, J. H., 404(5), 406(26), 413(26), *448*, *449*
Crowdy, S. H., 425(173), *453*
Cueto, C., 438(284), *455*
Cullis, W. C., 349(22), *392*
Cummings, H. W., 192(205), *219*
Curran, H. R., 14(37), *68*
Curran, P. F., 353(58, 60d), *393*

D

D'Abramo, F., 422(147), *452*
Dadd, R. H., 194(210), 207(241), 210(210), *219*, *220*, 385(244a), *400*
Daggs, R. G., 35(89), *69*
Dahlberg, L. Y., 90(186), *113*
Dahlqvist, A., 359(107), *395*
Dahm, P. A., 435(259), 438(280), 440(297, 298), 441(297, 298), *455*, *456*

Dale, W. E., 438(284), *455*
Daly, J. M., 89(157), *112*
Dam, H., 122(20), *214*
Danforth, W. F., 89(149a), *112*
Danielsson, H., 78(28), *108*, 383(236a), 384(236a), *400*
d'Arces, P. J., 363(129), *396*
Dark, F. A., 10(18), *68*
Darwin, C., 390(282), *401*
da Silva, A. C., 158(120), 187(120), *216*
Davenport, H. W., 353(60), 360(60), *393*, *401*
Davey, K. G., 269(181), *295*
Davidow, B., 411(69), 438(278), *450*, *455*
Davidson, W. D., 105(317), 107(317), *117*
Davies, A., 89(169), *112*
Davies, D. S., 325(114), 327(114), 330 (114), *343*
Davies, D. V., 356(83), *394*
Davies, F., 356(83), *394*
Davies, H. C., 320(95), *343*
Davies, R. E., 431(226), *454*
Davis, B. D., 85(130), *111*
Davis, G. K., 186(184), *218*
Davis, S. B., 325(114), 327(114), 330 (114), *343*
Davison, A. N., 411(63), *450*
Davson, H., 349(24), *392*
Dawes, B., 377(196), *398*
Dawson, A. B., 283(262), *297*
Day, H. G., 176(157), *218*
Day, M. F., 125(27), *214*, 382(231a), *399*
de Angelis, R. C., 158(120), 187(120), *216*
Deason, T. R., 88(147), *112*
de Boer, J. A., 269(175), *295*
DeCino, T. J., 411(71), *450*
Decker, G. C., 411(73), *450*
DeFloor, J., 84(109), *111*
DeFlora, A., 81(68), *109*
Defretin, R., 258(63), *292*
de Groot, A. P., 359(106), *395*
de Harven, E., 251(24), *291*
de Jong, M. I. C. P., 374(178), *398*
Dekker, C. A., 164(132), *217*
de Lerma, B., 268(165), *295*
DeLong, D. C., 149(94), 159(122), *216*, *217*
Del Vecchio, R. J., 228(49), *242*
DeMars, R., 102(285), *116*
deMeio, R. H., 421(152), *452*
Demerec, M., 156(113), *216*
de Metz, R., 382(231c), *399*
DeMoss, R. D., 85(125), *111*
Dennell, R., 235(101), *243*
Derrien, E., 422(143), *452*
Desnuelle, P., 105(314), *116*, 357(*see* footnote), *401*
Despopoulos, A., 442(311), *456*
Dethier, V. G., 355(74), *394*
Detweiler, D. K., 423(156), *452*
Deuchar, E. M., 59(176), *71*
Deutch, H. F., 50(149), *71*
Deutsch, V., 258 (*see* footnote)
Devigne, J., 386(256), *400*
Dewey, D. L., 155(112), *216*
Dewey, V. C., 147(87), 174(155), 175 (155), 180(155), *216*, *218*
de Wilde, J., 269(175), *295*
Diaz, J. C., 434(254), *454*
Dickens, F., 80(62), *109*
Dicker, S. E., 303(11), *340*
Dickman, A., 388(272), *401*
Dieke, S. H., 410(60), *450*
Dietrich, L. S., 92(200, 204), 93(200), *113*
Dill, W. A., 436(270), *455*
Dinning, J. S., 100(276), 101(276), *115*, 202(230), *220*
Ditzion, B., *401*
Dixon, G. H., 338(171), *345*
Dixon, H. B. F., 319(94), 320(97), 328 (127), 330(126, 128), *343*, *344*
Dixon, J. S., 319(85), 320(98, 99), 321 (85), 324(109), 325(99, 117), *342*, *343*
Dmitrieva, I. S., 356(86), 357(86), *394*
Dobreff, M., 374(180), *398*
Dobson, A., 362(124), 363(137, 138), *396*
Dobson, M. J., 362(124, 127), 363 (127), 364(142, 150d), *396*, *397*
Dodgson, K. S., 422(142), 423(142), *452*
Doi, R. H., 10(26), 11(25, 26), *68*
Dolphin, J. L., 61(179), *71*

Domnas, A. J., 89(171), 90(171), *112*
Dougherty, E. C., 121(2), 161(2), 188(188), 198(188), 199(188), 200(224), *213, 218, 219*
Dougherty, T. F., 92(211), 93(211), *113*
Drabkin, D. L., 90(183), *113*
Drihlon, A. C., 231(77), *242*
Drummond, G. I., 377(197), *398*
DuBois, K. P., 435(268), *455*
Duchâteau, G., 140(69, 70), *215,* 227(38, 40, 42), 228(45, 46), 231(69), *241, 242*
Duerksen, J., 89(164), *112*
Duerre, J. A., 84(107), 87(107), *110*
Dukes, H. H., 348(6), 357(6), 362(6), 367(6), 372(6), *392*
Dupont-Raabe, M., 263(131), 264(133), 268(164, 165), 270(164, 190b), *294, 295*
Durchon, M., 258(64, 67), 259(69, 71, 73, 75, 78), *292*
Durham, N. D., 84(98), 86(98), *110*
Durham, N. N., 413(85), *451*
Duspiva, F., 387(265), 388(273), *400, 401*
Dutton, G. J., 414(96), 416(116), 419(115, 116, 119, 120), 420(116, 119), 445(326), 448(116), *451, 456*
Duveau, J., 271(203), *296*
du Vigneaud, V., 304(22, 23, 24), 305(61), 307(29, 30), 309(22, 30, 44), 310(46–49, 53, 54), 311(24, 30, 56), 312(58), 313(61), 337(168), *341, 342, 345*

E

Eagle, H., 103(290), *116,* 161(125), 167(138), *217*
Eakin, E. A., 165(133), 173(148), 195(133), 196(217), *219*
Eakin, R. E., 196(217), 197(133), *217, 219*
Earle, N. W., 411(71), 440(307), *450, 456*
Easler, C. A., 359(108), *395*
Ebert, J. D., 57(167), *71,* 107(354), *118*
Echalier, G., 271(198, 199), *295*
Echols, H., 98(258), *115*
Eckstein, B., 107(339), *117*
Edwards, G., 251(24), *291*
Efimochkina, E. F., 424(170), 428(170), 431(170), *452*
Egelhaaf, A., 228(48), *242*
Eggleton, M. G., 303(11), *340*
Ehrhardt, P., 383(235a), *399*
Eigner, E. A., 325(114), 327(114), 330(114) *343*
Ekstrand, T., 415(129), *452*
Elam, D. W., 376(191), *398*
Elbers, P. F., 357(95), *395*
Ellinger, P., 425(183) 426(183), *453*
Elliott, A. M., 89(154), *112*
Elliott, S. D., 105(319), *117*
Elliott, W. H., 78(37), *108*
Elsden, S. R., 362(119), 363(119, 139), 366(119), *396*
Elton, R. L., 317(74), *342*
Elvehjem, C. A., 372(168), *397*
Engel, Chris, 351(37), 357(98), *393, 395*
Engel, S. L., 302(3), 303(3), 307(34), *340, 341*
Engelmann, F., 279(245), 282(245, 249), *297*
Engelsberg, E., 19(59), *69,* 84(99, 100, 101), 86(101), *110*
English, J. P., 320(95), *343*
Ennis, H. L., 25(74), 26(74), *69,* 105(325), 106(325), *117*
Ennor, A. H., 160(123), *217*
Enriques, P., 354(64), *394*
Entner, N., 82(77), *110*
Ephrussi, B., 19(58), 41(112), *69, 70*
Erspamer, V., 248(4), 255(53), 256(53), *291, 292*
Evans, D. R., 100(272), *115,* 355(74), *394*
Evans, W. C., 409(44), 436(44), *449*
Eyring, E. J., 76(9), 96(9), *108*

F

Fänge, R., 264(138), *294*
Fahraeus, G., 90(189), *113*
Failey, R. B., 424(178), *453*
Falk, J. E., 99(270), *115*
Fang, S. C., 418(104), *451*
Farber, E., 80(65), 81(65), *109*

Farmanfarmaian, S., 355(65a), *394*
Farner, D. S., 367(154a), 370(158, 161), 372(166), *397*
Faust, R. G., 25(75), *69*
Fawcett, C. H., 405(18), 406(19), *449*
Featherstone, R. M., 92(216), 94(216), *114*
Feigelson, P., 94(228–230), 104(228–230), 105(323), *114, 117*
Feldberg, W., 251(19, 20), *291*
Ferguson, W. C., 440(302), *456*
Fernandes, J. F., 166(136), *217*
Ferrando, R., 363(129), *396*
Ferreri, E., 384(239f), *400*
Fetcher, E. S., 352(55), *393*
Fiecchi, A., 407(40), *449*
Fielding, A. H., 435(265), *455*
Fildes, P., 79(47), *109*
Filosa, M. F., 25(75), *69*
Finamore, F. J., 232(85), *243*
Fincham, J. R. S., 150(98), *216*
Fineberg, R. A., 94(232), 106(326), *114, 117*
Finland, M., 404(3), *448*
Finn, B. M., 320(95), *343*
Fischer, F., 268(165a), 269(185, 187), 270(187, 190a), *295*
Fischer, G. A., 78(27), *108*
Fish, M. S., 411(65), *450*
Fisher, A. M., 332(137), 336(158), *344*
Fisher, H. A., 104(293), *116*
Fisher, J. R., 51(150, 151), *71*, 104(307), 105(307, 322), *116, 117*
Fisher, L. R., 122(13), 139(13), *214*
Fisher, R. B., 331(132), *344*, 349(25), 350(25), 352(57), 353(25), *392, 393*
Fishman, W. H., 419(113), *451*
Fitch, A., 337(168), *345*
Fitch, C. D., 100(276), 101(276), *115*
Fitch, W. M., 107(340), *117*
Fitzgerald, D. B., 84(110), *111*
Fitzgerald, R. J., 84(110), *111*
Fitz-James, P. C., 9(16), 18(50, 51), 19(50), *68*
Fitzpatrick, T. B., 318(79), *342*
Flaks, J. G., 107(343), *117*
Flannery, W. L., 137(60), 177(60), *215*
Fleischman, R., 103(290), *116*
Flickinger, R. A., 56(164), *71*
Fling, M., 89(176), 90(176), 99(176), 104(301), *112, 116*, 236(104), *243*
Florey, Elisabeth, 255(52), 256(52), *292*
Florey, Ernst, 255(52), 256(52), *292*
Florey, H. W., 372(170), *398*
Florkin, M., 126(33), 138(65), 139(65), 140(69, 70), *214, 215*, 227(34, 35, 38, 39, 40, 42, 43), 228(43, 45, 46), 231(69), *241, 242*, 283(264), *297*, 385(246a, 247), 388(274), *400, 401*, 414(97), *451*
Fogg, G. E., 137(56), 173(56), 183(177), *215, 218*
Folk, K. G., 29(81), *69*
Folkes, J. P., 82(72), *110*
Folley, S. J., 332(133), *344*
Follis, R. H., 186(182), *218*
Follis, R. H., Jr., 178(164), *218*
Fontaine, M., 284(267), *297*
Forbes, R. M., 363(131), *396*
Ford, D. L., 287(290), *298*
Forgash, A., 432(229), *454*
Forrest, H. S., 77(19), *108*
Foster, J. W., 5(8), 9(15), 11(28), 12(28), 14(35), 15(39), 18(35), 30(8), *67, 68*
Foster, M., 318(80), *342*
Fouts, J. R., 413(89), 446(330), *451, 456*
Fowden, L., 89(174), *112*
Fox, H. M., 99(264, 265, 266), 100(264), *115*
Fox, S. W., 130(43), 133(43), *215*
Foy, J. R., 433(238), *454*
Fraenkel, G., 156(114), 187(114), 192(202), 193(202), 198(114), 199(114), 205(114), 209(114, 251), *216, 219, 220*, 272(207), *296*, 378(212), *399*, 425(183), 426(183), *453*
Fraenkel, G. F., 209(250), *220*
Fraenkel, G. S., 205(234), *220*
Francis, J., 423(154), *452*
Frankel, D. L., 92(221), *114*
Frankel, D. R., 444(319), *456*
Franklin, T. J., 433(241), *454*
Franssen, J., *401*
Frazer, A. C., 351(39–41), *393*
Frech, M. E., 104(310), 105(310), *116*

Fredericq, E., 333(147), *344*
Freedland, R. A., 103(292), *116*
Freedman, J. K., 103(287), *115*
Freid, F., 264(134), *294*
French, C. S., 88(146), *112*
French, R. L., 412(80), *450*
Frenkel, A. W., 88(138, 139a), *111*
Freudenberg, E., 357(90c), 358(109), 359(90c, 109), *395*
Frey-Wissling, A., 413(90), 418(90), *451*
Fridhandler, L., 351(42), *393*
Fried, R., 158(120), 187(120), *216*
Frieden, E., 30(82), 56(165), 61(179, 180), *69*, *71*, 78(33), 104(304), *108*, *116*, 232(85), *243*
Friedler, L., 429(232), 432(232), *454*
Friedman, M., 76(11), *108*
Friedman, M. H. F., 370(163), 374 (176, 181), *397*, *398*
Friedmann, H., 372 (169a), 388(169a), *397*
Fritsch, R. H., 199(220), *219*
Fröhlich, F. W., 254(43), *291*
Fromageot, P., 304(25, 26), *341*
Fromme, I., 162(126), *217*
Fruton, J. S., 348(3), 356(3)
Fuhner, H., 441(308), *456*
Fuhrman, F. A., 353(59), *393*
Fujino, M., 336(162), *345*
Fukaya, M., 269(176, 177), *295*
Fukuda, S., 266(153), 269(169–171), 272(153, 209), *294*, *295*, *296*
Fukuda, T., 227(41), *242*
Fukuyama, T., 408(42), 423(159), *449*, *452*
Fuller, J. L., 359(108), *395*
Fuller, R. C., 84(94), 88(140), *110*, *111*
Fung, F. H., 444(316), *456*

G

Gabe, M., 260(91, 98–103), 261(102, 109, 110), 262(112, 115, 117), 266 (102), 271(196, 197), *292*, *293*, *295*
Gadaskin, I. D., 406(28), *449*
Gafford, R. D., 89(173), *112*
Gaffron, H., 88(136, 137), *111*
Gal, E. M., 444(316), *456*
Gale, E. F., 82(72), 84(95), *110*
Galstoff, P. S., 261(105), *293*
Galston, A. W., 90(186), 105(313), *113*, *116*, 122(9), 139(9), 189 (194), *214*, *219*
Gannon, N., 411(73, 74), *450*
Garcia, T. P., 434(255), *454*
Garen, A., 79(42), 97(42), 98(258), *109*, *115*
Garen, S., 98(258), *115*
Garrigus, U. S., 363(131), *396*
Garry, B. J., 76(14), 77(20), 96(20), 97(20), 98(20), *108*
Gaskell, J. F., 254(30, 31), *291*
Gaudette, L., 410(61, 62), 411(67), 446(67), 447(67), *450*
Gaue, A., 422(140), *452*
Gautrelet, J., 249(9), 250(9), *291*
Gee, L. L., 413(85), *451*
Geigy, R., 275(221), *296*
Geilenkirchen, W. L. M., 357(95), *395*
George, W. C., 384(237), *400*
Gerhart, J. C., 76(7), *108*
Gerischer-Mothes, W., 104(308), 105 (308), *116*
German, H. L., 372(169), *397*
Geronimus, L. H., 84(103), 86(103), *110*
Gersch, M., 268(158, 165a), 269 (182–185, 187), 270(182, 185, 187, 190a), *294*, *295*
Geschwind, I. I., 319(84, 92, 93), 320 (98), 321(84), 322(107), 324 (108), 325(117), 329, *342*, *343*
Gessner, T., 406(21), 407(21), 432 (234), 433(234), 436(21), *449*, *454*
Ghiretti-Magaldi, A., 39(100), *70*
Ghosh, N. C., 430(217), *454*
Giamician, G., 416(132), *452*
Giannotti, O., 411(70), *450*
Gibbs, M., 88(140), *111*
Gibor, A., 84(99, 100), *110*
Gibson, F., 96(247), *114*
Giese, A. C., 82(76), *110*, 386(252), *400*
Gilbert, L. I., 222(2a), 238(210), *241*, *243*, 267(156), 277(227), 278 (231a,b), 280(231, 231a), 281 (231b), *294*, *296*

Gilchrist, B. M., 99(266, 267), *115*
Gillette, J. R., 412(78), *450*
Gilmour, D., 107(334), *117*, 378(213c), 383(213e), *399*
Gish, D. T., 311(56), *342*
Giudice, G., 44(128), *70*
Glass, B., 107(328), *117*
Glazko, A. J., 436(270), *455*
Glick, D., 250(14), *291*
Glick, M. C., 191(199), *219*
Goehnauer, T. A., 288(300), *298*
Goffinet, B., 310(52), *341*
Goldman, M., 15(43), *68*
Goldsby, R. A., 88(139), 104(139), *111*
Goldsmith, E. D., 284(270), *297*
Goldsmith, T. H., 207(244), *220*
Goldstein, L., 102(286), *116*
Goodwin, T. W., 207(242), *220*, 263(125), *293*, 405(13), *449*
Goodyear, G. H., 170(146), *217*
Gorbman, A., 282(257), 284(266, 269), *297*
Gordon, A. S., 99(269), *115*
Gordon, H. T., 174(152), 177(152), *218*
Gordon, J. G., 365(149), *397*
Gordon, M. W., 92(222), *114*
Gordon, S., 304(22), 309(22), *341*
Gorini, L., 95(238, 239, 240), 97(240), 98(257a), 104(311), 105(325), 106(325), *114, 115, 116, 117*
Goswami, M. N. D., 92(210), 104(210), *113*
Gots, J. S., 413(86), *451*
Gould, E., 101(280), *115*
Gould, R. G., 101(277, 279), *115*
Grace, T. D. C., 125(27), *214*
Grady, A. B., 319(83), 320(83), *342*
Grassmann, W., 357(96), *395*
Gray, F. V., 363(132), *396*
Gray, H., 356(83a), *394*
Gray, J. L., 105(320), *117*
Grazi, E., 81(68), *109*
Green, H., 85(130), *111*
Green, J., 99(268), *115*
Green, J. P., 264(132), *294*
Green, J. R., 36(92), *69*
Green, S., 419(113), *451*
Greenbaum, A. L., 332(133), *344*
Greenbaum, L. M., 98(262), *115*
Greenberg, D. M., 92(205), 93(205), 94(232), 106(326), *113, 114, 117*, 427(196), 444(316), *453, 456*
Greenberg, J., 406(27), *449*
Greenfeld, L. J., 386(250), 387(250), *400*
Greengard, O., 94(228–230), 104(228–230), 105(321), *114, 117*
Greenstein, J. P., 2(1), *67*
Greer, M. A., 178(165), *218*
Gregerman, R. J., 254(33), *291*
Gregg, J. H., 22(65, 66), 23(65, 66), *69*
Gregor, H. P., 352(55), *393*
Gregory, R. A., *401*
Grelet, N., 18(49), *68*
Grenby, T. H., 433(244), *454*
Grieg, C. G., 414(96), *451*
Griffith, T., 158(121), *217*
Grobe, B., 275(221), *296*
Gröschel, U., 235(102), *243*
Gromadska, M., 231(69), *242*
Gros, P., 92(197), 94(197), *113*
Gross, D., 93(226), *114*
Gross, S. R., 89(173), *112*
Grossman, D. J., 362(117), 367(117), *396*
Grossman, M. I., 348(13a), *392, 401*
Grove, J. F., 435(266), *455*
Grützner, P., 358(99), *395*
Grunicke, H., 81(65a), *109*
Guillemin, R., 307(28), 317(76), 319(88), *341, 342, 343*
Guirard, B. M., 166(134), *217*
Gundersen, W., 98(257a), *115*
Gunn, D. L., 205(234), *220*
Gurewitsch, I. I., 406(28), *449*
Gustafson, T., 45(135), 46(135, 137), 50(149), 51(137, 152), *70, 71*
Guthrie, F. E., 412(79), *450*
Guttman, B., 264(134), *294*
Guttmann, S., 310(50), 321(102, 103, 104), 322(103, 104), 327(119, 120), 328(102, 125), 330(125), *341, 343, 344*
György, P., 191(199), 197(218), *219*

H

Hackman, R. H., 229(54), 234(98), *242, 243*

Hackney, A. L., 22(65), 23(65), *69*
Hadorn, E., 229(56), *242*
Hagadorn, I. R., 260(90), *292*
Hagan, J. J., 105(316), *117*
Hageman, R. H., 90(188), *113*
Hahn, F. E., 82(73), *110*
Halcro-Wardlaw, H. S., 35(89), *69*
Haldane, J. B. S., 129(41), *214*
Hale, W. H., 363(131), *396*
Hall, R. J., 176(158), *218*
Halliburton, W. D., 349(22), *392*
Halliwell, G., 362(121), *396*
Halpern, Y. S., 96(249), *114*
Halpin, J. G., 372(168), *397*
Halver, J. E., 149(94), 159(122), *216, 217*
Halvorson, H., 5(4), 7(11), 8(14), 10(26), 11(25, 26), 15(4), 17(4, 11), 18(11), 23(68), *67, 68, 69*
Halvorson, H. O., 5(5), 11(27), 12(29), 13(29), 16(5, 29, 45), 19(55), *67, 68, 69,* 89(162–166, 179), 90(162, 179, 181, 181a), 93(179), *112, 113*
Hampp, E. G., 196(215), *219*
Hamburger, K., 282(251), *297*
Hanby, W. E., 150(97), *216*
Hand, D. B., 406(23), *449*
Handler, P., 79(38c), *109*
Hannig, K., 357(96), *395*
Hansen, E. L., 188(188), 198(188), 199(188), 200(224), *218, 219*
Hanser, G., 275(214, 216), 276(216), *296*
Hanström, B., 263(127), 270(188), *294, 295*
Hardwick, W. A., 9(15), 15(39), *68*
Harfenist, E. J., 333(141, 145, 146), 336 (159), *344*
Harker, J. E., 269(174), *295*
Harms, J. W., 258(60), 259(60), 260(60), *292*
Harms, W. R., 260(96), *293*
Harris, B., 364(143), *396*
Harris, D. L., 55(161), *71*
Harris, G., 90(184), *113*
Harris, G. W., 303(9), *340*
Harris, J. F., 409(43), *449*
Harris, J. I., 320(98), 321(101), 322(106), 324(110), 325(116, 117), 336(161, 165), 337(165), *343, 345*
Harris, L. E., 364(150c), *397*
Harris, P. L., 192(206), *219*
Harris, R. S., 122(18), 139(18), *214*
Harrison, A., 438(289), *455*
Harrow, B., 348(1), 356(1), 357(1), *392*
Hartman, P. E., 156(113), *216*
Hartree, E. F., 10(19), *68*
Harvey, W. R., 224(27, 29, 30), 225(27, 29, 30), *241*
Hasegawa, K., 269(168, 172, 173), *295*
Haslewood, G. A. D., 351(35, 36), *393,* 431(228), *454*
Hasselberg, I., 51(152), *71*
Hassett, C. C., 355(76), *394*
Hatch, M. D., 80(60), *109*
Hauenschild, C., 258(66), 259(70, 72, 74, 79), *292*
Haurowitz, F., 376(186), *398*
Hawkes, L. E., 122(7), 139(7), *214*
Hawking, F., 423(153), *452*
Hawthorne, J. N., 210(256), *220*
Haydak, M. H., 288(300), *298*
Hayes, F. R., 41(113), *70*
Hayes, H. H., 364(144), *396*
Hayes, W. J., 438(288), *455*
Heatley, N. G., 54(160), *71*
Hebb, C. O., 251(18, 21), *291*
Hecker, E., 286(277, 279, 280, 282), *298*
Hein, R. E., 435(259), *455*
Hele, M. P., 350(32), *393*
Helfand, M., 427(197), *453*
Heller, H., 302(1), 303(16), 308(40), 312(40, 57), 314(1), *340, 341, 342*
Heller, J., 232(78, 78a), *243,* 432(230), *454*
Henderson, F. G., 424(178), *453*
Henderson, L. J., 134(48), *215*
Henderson, L. M., 158(121), *217*
Henis, Y., 84(108), *111*
Henry, K. M., 367(155), *397*
Heppel, L. A., 79(45), *109,* 437(275), 439(275), *455*
Herbst, C., 45(130), *70*
Herbst, E. J., 210(257), *220*

Herlant-Meewis, H., 260(82, 84), 261 (111), *292, 293*
Herner, A. E., 56(165), *71*
Herriott, R. M., 104(299), *116*, 356 (88, 93b), 357(88), 376(88), *394, 395*
Hess, G. P., 311(56), *342*
Hestrin, S., 419(112), *451*
Hewitt, E. A., 370(159), *397*
Hewitt, E. J., 90(190), 91(190), *113*
Heymes, R., 310(52), *341*
Hiatt, H. H., 104(296, 297), 105(297), 106(296), *116*
Hickey, M. D., 98(262), *115*
Hidaka, T., 234(94), *243*
Higgins, E. S., 413(88), *451*
Hightower, N. C., Jr., 348(10a), *392*
Hilchey, J. D., 150(96), *216*
Hildebrandt, A. C., 125(28), *214*
Hill, R. L., 337(167), *345*
Hilz, H., 422(148), *452*
Himwich, W. A., 434(249), *454*
Hinman, R. L., 90(181), *113*
Hirata, Y., 269(173), *295*, 429(215), 430(215), *454*
Hiratani, T., 427(200), *453*
Hirsch, G. C., 355(72), 376(185), 378 (201), 380(201), 381(201), 384 (201), 385(240), 389(201), *394, 398, 400*
Hisaw, F. L., 283(262), *297*
Hitchings, G. H., 78(24), *108*
Hobson, A. D., 254(39), *291*
Hobson, R. P., 388(267, 268), *401*
Hodge, M., 262(118), 263(118), *293*
Höber, R., 350(33), 353(33), *393*
Hörstadius, S., 45(131, 132), 48(144), *70, 71*, 381(225), *399*
Hörstadius-Kjellström, G., 381(225), *399*
Hoffmeister, H., 274(212a, 213c), *296*
Hofmann, A. F., 351(38a), 384(38a), *393*
Hofmann, H., 385(246), *400*
Hofmann, K., 321(105), 322(105), 327 (122), 328(122, 124), 330(122), *343, 344*
Hofsten, B. V., 89(168), *112*
Hogben, L. J., 254(39), *291*
Hogg, J. F., 89(153a, 154), *112*
Hogness, D. S., 83(85), *110*
Holaday, D., 170(146), *217*
Holden, M., 386(260), *400*
Hollande, H. A.-C., 405(7), *448*
Holley, R. W., 406(23), *449*
Holmes, W. N., 318(77), 330(77), *342*
Holmsen, J., 420(138), *452*
Holter, H., 47(138), *70*, 390(283), *401*
Holton, P., 303(6), *340*
Holzer, H., 81(65a), *109*
Honzl, J., 310(51), *341*
Hopkins, T. L., 435(260), *455*
Hoppe, W., 274(212a), *296*
Hopps, H. E., 82(73), *110*
Horie, J., 213(74), 232(79), *242, 243*
Horiuchi, S., 96(253), *115, 401*
Horiuchi, T., 96(253), *115*
Horn, N. L., 31(84), *69*
Horning, E. C., 411(65), *450*
Horowitz, N. H., 89(176), 90(176), 99 (176), 104(301), *112, 116*, 236 (104), *243*
Horsfall, J. G., 406(22), 435(267), *449, 455*
Horwitt, M. K., 192(201), *219*
Hoshiai, Z., 425(182), *453*
Hoskins, W. M., 439(292), 440(292, 306), *455, 456*
House, H. L., 153(102), 167(137), 174 (137, 151), 199(151), 205(151), *216, 217, 218*, 378(213a,b), *399*
Houssay, B. A., 331(129), *344*
Howard, A. N., 317(73), *342*
Howard, K. S., 320(95), 325(114), 327 (114), 330(114), *343*
Howden, G. F., 231(68), *242*
Howes, N. H., 207(239), *220*
Hsia, D. Y., 107(335), *117*
Hsu, C., 100(276), 101(276), *115*
Huang, I., 107(335), *117*
Huang, K. C., 442(311), *456*
Huber, R., 274(212a), *296*
Hubl, H., 260(81, 88), *292*
Hübscher, G., 210(256), *220*
Hucker, H. B., 412(78), *450*
Huf, E., 349(19), *392*
Huffman, C. F., 362(120), 363(120), 366(120), *396*
Hughes, H. B., 424(164), *452*

Huguenin, R. L., 312(59), 321(104), 322(104), 328(125), 330(125), *342, 343, 344*

Huguley, C. M., 78(25), 102(25, 283a), *108, 115*

Hultin, T., 42(122, 123), 43(123, 126), 44(129), *70*

Humphris, B. G., 422(144), 442(144, 312), *452, 456*

Hundley, J. M., 187(186), 188(186), *218*

Hungate, R. E., 364(142), 387(261, 262), *396, 400*

Hunter, J. R., 8(12), *67*

Hutchens, J. O., 47(140), *70*

Hutchinson, A., 416(107), 417(133), 418(103, 107), 419(107), *451, 452*

Hutchinson, G. E., 127(35), *214*

Hutner, S. H., 88(143, 144), 89(152), *111, 112,* 151(100), 166(100), 174(150), *216, 217*

Hyatt, M. T., 19(57), *69*

Hyman, L. H., 380(223), *399*

I

Ichikawa, M., 268(157), *294*

Idler, D. R., 448(337), *457*

Ihnen, K., 368(157), *397*

Immers, J., 41(111), *70*

Imsande, J., 79(38c), 96(245), *109, 114*

Irzykiewicz, H., 389, *401*

Iselin, B., 324(112), *343*

Isenberg, H., 202(232), *220*

Ishida, J., 376(188), *398*

Ishihara, Y., 336(162), *345*

Ishizaki, H., 268(157), *294*

Ito, T., 224(7, 8), 231(74), 232(79), *241, 242, 243*

Ito, Y., 336(162), *345*

Ivanov, N., 354(62), *394*

Ivy, A. C., 102(282), *115*

Iwasaki, T., 429(215), 430(215), *454*

Iyama, Y., 447(333), *457*

J

Jackson, C., 90(181a), *113*

Jackson, M., 19(55), *69*

Jacob, F., 96(246), 97(246, 257), *114, 115*

Jacobs, M. H., 42(124), *70*

Jacobson, M., 122(21), *214,* 286(283, 284), 287(284a, 286b), *298*

Jailer, J. W., 317(71), *342*

James, A. L., 31(86), 32(86), 33(86), *69*

James, F., 364(143), *396*

James, M. F., 363(131), *396*

James, W. O., 31(86), 32(86), 33(86), *69*

James, S. P., 423(163), 433(239), *452, 454*

Janssen, B. C. P., 195(212), *219*

Jaquenoud, P. A., 310(50), 321(104), 322(104), 327(119, 120), 328(125), 330(125), *341, 343, 344*

Jard, S., 304(20), 305(20), *340*

Jeanloz, R. W., 162(128), *217*

Jenkins, G. N., 169(144), *217*

Jennings, J. B., 381(220a)

Jensen, D., 92(205), 93(205), *113*

Jensen, H., 105(320), *117*

Jensen, J. A., 438(284), 439(296), 440(296), *455, 456*

Jensen, W. A., 90(187), *113*

Jepson, J. B., 427(199), *453*

Jervis, E. L., 352(56), *393*

Jeuniaux, Ch., 84(114), *111,* 227(38, 42), *241, 242,* 361(116, 116a), 366(151), 367(153a), 372(116a), 373(171), 382(231c), 385(248, 249), 386(253–256, 258), *396, 397, 398, 399, 400, 401*

Jezewska, M., 232(78, 78a), *243,* 432(230), *454*

Johl, A., 312(58), *342*

Johnson, A. W., 415(129), *452*

Johnson, B. C., 426(184, 185), 427(204), 428(204), 429(204), *453*

Johnson, F. R., 352(56), *393*

Johnson, N. M., 411(65), *450*

Johnson, W. J., 424(165), *452*

Johnston, N. C., 288(295, 296), *298*

Johnston, T. B., 356(83), *394*

Joliffe, N., 413(87), *451*

Joly, L., 234(93), *243,* 279(237), *297*

Joly, P., 234(93), *243*

Jondorf, W. R., 411(66), 414(100), 420

(123, 139), 421(139), 445(100), 446(100), *450, 451, 452*
Jones, C. R., 438(289), *455*
Jones, D. R., 425(173), *453*
Jones, I. C., 318(77), 330(77), *342*
Jones, M., 78(23), 92(216), 94(216), *108, 114*
Jones, W. A., 122(21), *214,* 286(283), 287(284a), *298*
Jordan, E., 84(116), *111*
Jordan, H. J., 349(17), 354(17, 63), 355 (17, 63), 378(201, 214), 380(201), 381(201), 383(214), 384(201), 389(201), *392, 394, 398, 399*
Josephson, E. S., 406(27), *449*
Josse, J., 107(344), *117*
Jütting, G., 196(216), *219*
Julian, J. R., 386(251), 387(251), *400*
Julin, C., 283(260), *297*
Jull, J. W., 407(32), *449*
Jullien, A., 250(11, 16), 254(44), *291*
Junold, J., 374(179), *398*
Jurišić, P. J., 349(18), *392*

K

Kaars Sypesteyn, A., 364(140), *396*
Kalckar, H. M., 84(116), *111*
Kalf, G. F., 231(67, 70), *242*
Kallio, R. E., 85(126), 93(126), *111*
Kalmus, H., 264(139), *294*
Kaltenbach, J. C., 41(116), *70*
Kamaki, S., 447(332), *456*
Kapitza, W., 268(165a), 270(190a), *295*
Kappeler, H., 324(112), 327(122a), *343, 344*
Kaplan, N. O., 104(310), 105(310), *116*
Karkhanis, Y., 104(304), *116*
Karlson, P., 104(303), 107(303), *116,* 223, 227(33a), 229(55), 233(89), 234(99, 100), 235(55, 103, 103a, b), 236(103, 105), 237(103, 103a, 108), 238(111), 239, *241, 242, 243,* 265(141), 268(141), 270(190), 271(200, 201), 272(141, 206), 273 (141), 274(212a,b, 213, 213b,c), 275(141, 214–217, 220–222), 276 (215, 216, 220), 277(222, 229), 278(229, 231c, 232), 279(247), 281(231c), 282(232, 247), 285 (275, 276), 287(276, 289), 290 (304), *294, 295, 296, 297, 298*
Karrer, P., 385(246), *400*
Karvinen, E., 102(282), *115*
Kato, M., 426(187), *453*
Kato, Y., 92(217), 94(217), *114*
Katsoyannis, P. G., 305(61), 310(48, 49), 311(56), 313(61), *341, 342*
Kattermann, R., 90(182), 104(182), *113*
Katz, B., 254(50), *292*
Kaufmann, S., 156(115), *216*
Kavanau, J. L., 41(119, 120), 42(120), *70*
Ke, Y. H., 413(85), *451*
Kearns, C. W., 437(276), 439(293, 295), 400(302, 304, 305), *455, 456*
Keech, D. B., 84(90), 86(90), *110*
Keilin, D., 10(19), *68*
Kellerman, G. M., 430(222), *454*
Kelsey, F. E., 412(76), *450*
Keltch, A. K., 47(140), 48(145), 49 (145), 50(147), *70, 71*
Kemp, P., 210(256), *220*
Kennedy, E. P., 79(38b), *109*
Kenney, F. T., 94(231), *114,* 445(322), *456*
Kent, P. W., 415(126), *451*
Kenten, R. H., 405(10), *449*
Kenyon, W. A., 373(173), *398*
Keosian, J., 133(47), *215*
Kern, J., 372(169a), 388(169a), *397*
Kerr, N. S., 20(63), *69*
Kershaw, T. G., 351(50), *393*
Kersten, H., 194(209), *219*
Kersten, W., 194(209), *219*
Ketterer, B., 302(2), *340*
Khalil, F., 428(207–210), 430(209), *453*
Kidder, G. W., 122(4), 147(87), 166 (4), 174(155), 175(155), 180 (155), *213, 216, 218,* 378(205), *398*
Kikal, T., 413(82), *450*
Kikkawa, H., 158(119), *216,* 415(128), 429(214, 215), 430(214, 215), *451, 454*
Kilby, B. A., 231(68), *242*
Kilian, E. F., 380(220), 381(220), *399*
Kingscote, A. A., 444(315), *456*

Kinoshita, S., 77(17a), *108*, 155(111), *216*
Kinoshita, T., 424(166), *452*
Ki Paik, W., 62(183), 67(183), *71*
Kirimura, J., 267(155, 155a,b), *294*
Kirkwood, J. G., 333(142), *344*
Kistner, A., 145(82), *216*
Kitada, S., 155(111), *216*
Kitai, R., 334(155, 157), 336 (157, 160), *344*
Kizimura, T., 227(41), *242*
Klee, P., 358(102), *395*
Klein, E., 92(212, 213), 94(212, 213), *113, 114*
Kleinholz, L. H., 263(124), 264(124), *293*
Kleinzeller, A., 349(14b), 353(14b), *392*
Klingenberg, M., 187(185), 191(185), *218*
Klingmuller, V., 431(224), *454*
Klok, J. H., *401*
Klopotowski, T., 77(19a), *108*
Knappe, J., 196(216), *219*
Knight, D. R., 372(169), *397*
Knight, R. H., 433(237), *454*
Knoefel, P. K., 442(311), *456*
Knowles, F. W. G., 262(120), 263(122, 123, 131), 264(133), 268(165), 270(190b), *293, 294, 295*
Knox, W. E., 92(196, 198, 202, 203, 207–210), 104(210), 107(198, 342), *113, 117*, 357(86a), *394*
Kobayashi, M., 231(75), *242*, 260(94), 266(154), 267(155, 155a,b), 284 (266), *293, 294, 297*
Koch, H., 269(187), 270(187), *295*
Kogut, M., 85(127), *111*
Koller, G., 260(95), 269(178, 179), *293, 295*
Kolpech, G., 125(26), *214*
Kolderie, M. Q., 45(134), *70*
Kolesnikov, P. A., 31(87), *69*
Komori, Y., 425(181), 428(181), 429 (181), 430(181), *453*
Konigsberg, N., 88(141), *111*
Kopeč, S., 266(145), *294*
Korjuijeff, P. A., 360(113), 371(165), *395, 397*
Korkes, S., 84(104), *110*
Kornberg, A., 78(35), 107(344), *108, 117*
Kornberg, H. L., 35(90), *69*, 79(51), 81(66), 84(66, 89, 94), 89(153a), 96(66), 99(263), *109, 110, 112, 115*
Kornberg, S. R., 107(344), *117*
Korner, A., 332(134), *344*
Koschtojanz, C. S., 360(113), 371(165), *395, 397*
Kostellow, A. B., 92(219, 220), 94(219, 220), *114*
Kosto, B., 318(80), *342*
Kotake, Y., 231(73), *242*
Kotyk, A., 349(14b), 353(14b), *392*
Koyama, J., 104(312), *116*
Kozloff, L. M., 83(83), 103(83), 105 (83), *110*
Krahl, M. E., 38(98), 41(109), 47(140, 141), 48(145), 49(145, 156), 50 (146, 147), *70, 71*
Krakowski, M. C., 103(292), *116*
Kramer, M., 86(121), *111*
Krane, S. M., 47(142), 48(142), *70*
Krasna, A. I., 104(293, 294), *116*
Kratzer, F. H., 155(110), *216*
Krebs, H. A., 79(51), *109*, 423(157), *452*
Kretschmer, N., 445(322), *456*
Krichevsky, M., 27(76), *69*
Krijgsman, B. J., 380(222), 389(279), *399, 401*
Krishna, S. S., 383(235b), *400*
Kritchevsky, D., 101(278), *115*
Krivanek, J. O., 22(65), 23(65), 28(78), *69*
Krivanek, R. C., 28(78), *69*
Krogh, A., 223(3), *241*
Krueger, H. R., 443(313), *456*
Kruse, I., 122(20), *214*
Kruta, V., 254(38), *291*
Kühn, A., 266(146–148), *294*
Kühn, A. Z., 228(47), *242*
Kühne, H., 262(113), *293*
Kunitz, M., 104(299), *116*, 356(88), 357(88), 376(88), *394*
Kunkee, R., 83(82), *110*
Kuo, M. H., 96(254), 99(254), *115*
Kurahashi, K., 84(116), *111*

Kurland, C. G., 10(24), 57(170), *68, 71*, 225(31), *241*
Kusui, K., 404(6), *448*
Kylä-Siurola, A.-E., 79(43), 98(43), *109*

L

Ladd, J. N., 201(227, 228), 202(229), *220*
LaDu, B. N., 410(61), *450*
Lande, S., 327(122), 328(122), 330(122), *344*
Landgrebe, F. W., 302(2), 303(8, 14), 318(78), 319(89, 90), *340, 342, 343*
Landman, O. E., 89(167), *112*
Landmann, W. A., 325(115), *343*
Lane, C. E., 386(250), 387(250), *400*
Lang, K., 434(248), *454*
Langdon, R. G., 338(169), *345*
Lanzavecchia, G., 104(311), *116*
Lara, F. J. S., 84(111), *111*
Lardy, H. A., 79(48), 107(347), *109, 118*, 356(86b), *394*
Larminie, H. E., 99(270), *115*
Larsen, A. D., 85(126), 93(126), *111*
Larsen, H., 142(73), *215*
Larsen, J. R., 279(246), *297*
Lascelles, J., 88(149), 96(252), 100(271), *112, 115*
Lasker, R., 386(252), *400*
Laskowski, M., 104(309), *116*
Laszt, L., 350(31), *393*
Laties, G. G., 57(171), *71*
Laufer, H., 229(58), *242*
Laug, E. P., 438(291), *455*
Laverack, M. S., *401*
Laviolette, P., 271(191), *295*
Law, L. W., 166(135), *217*
Lawler, H. C., 304(24), 310(54), 311(24), *341, 342*
Lawrence, A. L., *401*
Lawrence, J. S., 423(153), *452*
Lawrence, N. L., 8(13), *67*
Laws, B. M., *401*
Layton, L. L., 444(319), *456*
Lea, A. S., 356(84a), 360(110), *394, 395*
Le Bars, H., 364(141), *396*
Leclercq, J., 140(69), *215*
Lederer, E., 288(297), *298*
Lederberg, J., 123(24), 134(49), 135(49), *214, 215*
Lee, N. D., 92(199), *113*
Lee, T. H., 318(81), 319(81, 82, 86), 320(100), 321(86), 324(86, 109), 325(100), *342, 343*
Lee, Y.-P., 107(347), *118*
Lees, A. D., 266(144), 269(144), *294*
Lees, H., 143(76), *215*
Lehman, A., 438(278), *455*
Lehman, R. L., 355(71), *394*
Lehninger, A. L., 41(110), *70*
Lelouchier-Dagnelie, H., 96(243), *114*
Leloup, J., 284(267), *297*
Lenci, M. T., 307(31, 32, 36, 37), 308(38), 310(31, 32), 311(31, 32), 312(37, 38), 313(37), 314(65), *341, 342*
Lender, T., 257(56, 57), 258(*see* footnote), *292*
Lenicque, P., 45(135), 46(135, 137), 51(137), *70*
LePage, G. A., 78(22, 23), *108*
Lerner, A. B., 318(79, 81), 319(81, 86), 320(100), 321(86), 324(86, 109), 325(100), *342, 343*
Lester, D., 208(247), *220*
Lester, G., 77(18), 98(18), *108*
Letellier, A., 430(216), *454*
Lev, M., 191(200), *219*
Levenbook, L., 232(84), *243*
Levin, A. P., 86(117), 96(244), 105(117), *111, 114*
Levine, E. P., 138(64), 185(181), *215, 218*
Levine, H., 178(163), *218*
Levine, M., 93(225), *114*
Levinson, H. S., 5(6, 9), 6(9, 10), 7(6), 9(6), 19(57), *67, 69*
Levinson, Z. H., 432(231), *454*
Levinthal, C., 79(42), 97(42), *109*
Levy, A. L., 320(98), *343*
Levy, J. B., 19(59), *69*, 84(99, 100, 101), 86(101), *110*
Lewin, J. C., 88(145), *112*
Lewin, R. A., 88(139a, 145), *111, 112*
Lewis, D., 362(118a), 363(118a, 130), 365(118a, 150g), 366(118a), *396, 397*
Ley, J., 84(109), *111*

L'Hélias, C., 231(76), *242*, 268(160, 161), 270(161), 282(252, 256), *294, 297*
Li, C. H., 319(85, 92, 93), 320(98, 99), 321(85), 322(107), 324(108, 111), 325(99), 325(117, 118), 327(121), 328(121, 123), 329, 336(165), 337 (165), *342, 343, 344, 345*
Lichstein, H. C., 84(107), 87(107), *110*
Liddel, G. U., 21(64), 22(64), 25(64), 26(64), 27(64), *69*
Liebau, H., 236(105), *243*
Lieberman, I., 78(35), 104(298), *108, 116*
Light, A., 307(29, 30), 309(30), 311 (30), *341*
Lilly, V. G., 122(8), 139(8), *214*
Lin, E. C. C., 86(117), 92(198, 207, 208, 209), 105(117), 107(198), *111, 113*, 357(86a), *394*
Lin, P. H., 426(184, 185), *453*
Lin, T. M., 102(282), *115*
Lindahl, P. E., 45(136), 47(136, 138), 54(160), *70, 71*, 445(323, 324, 325), *456*
Linday, E. M., 84(91), 86(91), 104 (91), *110*
Linderstrøm-Lang, K., 387(265), 390 (283), *400, 401*
Lindquist, B., 169(144a), *217*
Lindquist, D. A., 438(280), 440(298), 441(298), *455, 456*
Linnemann, G., 390(284), *401*
Linzen, B., 233(90), *243*
Lipke, H., 156(114), 187(114), 198 (114), 199(114), 205(114), 209 (114), *216*, 378(212), *399*, 439 (295), *456*
Lipmann, F., 80(59), 81(59), *109*, 145 (78), *215*, 422(145–148), *452*
Lipscomb, H. S., 307(28), 317(76), *341, 342*
Liss, M., 98(262), *115*
Litchfield, J. B., 42(125), *70*
Litchfield, J. T., 424(168), *452*
Littlejohn, J. M., 188(191), *219*
Liu, T. Y., 327(122), 328(122), 330 (122), *344*
Ljunggren, H., 90(189), *113*
Lloyd, F. E., 390(285), *401*
Lo, T. B., 327(121), 328(121), *343*
Lockwood, W. W., 337(168), *345*
Løvtrup, S., 52(156), 53(158), 54 (158), 58(172), 59(172), *71*
Lonberg-Holm, K. K., 79(50), *109*
Long, J. M., 319(88), *343*
Loo, T. L., 31(83), *69*
Loomis, W. F., 181(172), 195(172), *218*
Lorch, E., 196(216), *219*
Loud, V., 101(280), *115*
Loughheed, T. C., 227(37), 231(37), *241*
Lovatt Evans, C., 348(5), 357(5), *392*
Lovett, J. S., 24(72), *69*, 107(329), *117*
Low, E. M., 208(248), *220*
Lowartz, C., 383(233), *399*
Lozet, F., 385(247), 388(274), *400, 401*
Lubet, P., 261(107), *293*
Lucarain, C., 304(18), *340*
Luderitz, O., 162(126), *217*
Ludwig, D., 224(10–16), 226(32), 228 (51–53), 231(10), 232(51), *241, 242*
Lüscher, M., 275(220), 276(220), 279 (239, 241), 282(249), 285(275), 287(293), 288(239, 241, 298, 299), *296, 297, 298*
Lukton, A., 152(101), 206(101), *216*
Lundh, G., 359(107), *395*
Lutz, B. R., 254(49), *292*
Luypen, A. F. M. G., 376(184), *398*
Luzzati, M., 77(19a), *108*
Lwoff, A., 88(142), 89(151, 152), *111, 112*, 122(11), 139(11), 166(11), 190(195), 193(195), 194(195), 209(195), *214, 219*, 378(208a), *399*
Lyman, C. M., 170(146), *217*
Lynen, F., 196(216), *219*, 425(175), *453*

M

Maas, W. K., 95(238), *114*
Macallum, A. B., 135(54), *215*
McAnally, R. A., 362(118, 123), 363 (118), 365(118), 366(118), *396*
Macaulay, M. H. F., 303(8), *340*
McCarter, J. R., 191(198), *219*
McConnell, K. P., 427(193), *453*

Macdonald, A. J., 367(155), *397*
MacDonald, S. F., 415(129), *452*
McDougall, E. J., 349(14), 350(14), 351(14), 353(14), *392*
McElroy, W. D., 107(328), *117*
McEnroe, W. D., 432(229), *454*
McFall, E., 102(284), *115*
Macfarland, R. H., 435(259), *455*
McGarrahan, K., 101(280), *115*
McGilvery, R. W., 105(317), 107(317), *117*
McGinnis, A. J., 153(103), *216*
McGirr, J. C., 438(289), *455*
McGregor, R. M., 319(87), *342*
Mackinney, G., 152(101), 206(101), *216*
McLean, M. J., 208(247), *220*
Maclean, N. M., 254(41), *291*
MacLeod, C. M., 103(287a), 107(349), *116, 118*
MacLeod, H. L., 89(176), 90(176), 99(176), *112*
MacMillan, A. L., 193(208), *219*
McNeill, G., 67(184), *71*
Maetz, J., 304(18, 20), 305(20), 308(38), 312(38), 316(68), *340, 341, 342*
Magasanik, B., 18(48), 19(48), *68*, 75(3), 76(13), 86(117), 96(244), 102(284), 105(117), *107, 108, 111, 114, 115*
Magee, D. F., 364(150b), *397*
Magee, H. E., 367(155), *397*
Maggio, R., 39(100), *70*
Magrath, D. I., 160(123), *217*
Maickel, R. P., 411(66, 67), 414(100), 420(123, 139), 421(139), 445(100), 446(67, 100), 447(67), *450, 451, 452*
Maier-Huser, H., 304(25, 26), *341*
Maki, Y., 446(331), 447(332), *456*
Makino, K., 424(166), *452*
Maley, F., 107(336), *117*
Maley, G. F., 107(336), *117*
Manchester, K. L., 332(134), *344*
Mandels, M., 84(115), 89(115, 158), 90(115), *111, 112*
Mandelstam, J., 13(31), 14(32), 18(53), 23(69), *68, 69*, 86(118), *111*
Manegold, J. H., 154(106), *216*
Mangan, J. L., 365(150), *397*
Mangold, E., 358(101), 360(101), 361(101), 362(101), 365(101), 367(101), 368(101), 370(101), *395*
Manis, J. G., 427(205), 428(205), 431(205), *453*
Mann, T., 155(109), *216*
Mansour, K., 384(239a), 387(263), *400*
Mansour-Bek, J. J., 378(206), 382(232), 383(206), 384(239b), 387(263), *398, 399, 400*
Mantica, E., 162(127), *217*, 415(125), *451*
Mapson, L. W., 420(122), *451*
March, R. B., 411(70), *450*
Marcus, A., 38(95), *69*, 107(331), *117*
Marcus, L., 84(96), *110*
Margolis, L., 376(192), *398*
Marnay, D., 250(13), *291*
Marr, A. G., 84(96), *110*
Marsh, C. A., 413(92), *451*
Marshall, E. K., 423(155, 158), *452*
Marshall, M., 62(182, 183), 67(183), *71*, 107(351), *118*
Marshall, S. M., 378(209), *399*
Martin, A. W., 80(63), *109*
Martin, R. G., 76(14), *108*
Mason, H. S., 406(20), *449*
Masson, M. J., 363(133), *396*
Mast, S. O., 387(262a), *400*
Mathews, H., 61(180), *71*
Mathies, J. C., 376(192), *398*
Matsubara, H., 435(262), *455*
Matthes, D., 287(289a), *298*
Matthews, J., 353(60c), *393*
Matthews, R. E. F., 149(93), *216*
Mattson, A. M., 411(72), 437(274), 438(284, 286, 287, 288), *450, 455*
Mattson, F. H., 208(245), *220*
Maurer, R., 274(213b), *296*
Maurer, R. L., 176(157), *218*
Maurice, A., 354(61), *393*
Mayer, R. L., 409(45), *449*
Maynard, L. A., 122(16), 139(16), *214*
Mazur, A., 348(1), 356(1), 357(1), *392*
Meadow, P. M., 85(128), *111*
Mehl, J. W., 40(108), *70*
Meienhofer, J., 327(121), 328(121), *343*

Meisenhelder, J. H., 320(95), *343*
Meiss, A. N., 90(192), *113*
Meister, A., 430(221), 431(221), *454*
Mennega, A. M. W., 359(104), 370 (104), *395*
Mercer, E. H., 382(231a), *399*
Merritt, C., Jr., 287(286a), *298*
Merten, R., 357(96), *395*
Mertz, E., 159(122), *217*
Mertz, E. T., 149(94), *216*
Metcalf, R. L., 410(58), 411(70), *450*
Metzenberg, R. L., 62(183), 67(183), *71*, 107(351), *118*
Miahle, P., 332(135), *344*
Michl, H., 309(45), *341*
Mikhlin, D. M., 31(87), *69*
Militzer, W., 10(23), *68*
Miller, D., 353(60a), *393*
Miller, E. C., 92(206), *113*
Miller, G. G., 287(285, 286), *298*
Miller, J. A., 92(206), *113*
Miller, L. L., 357(97), *395*
Miller, L. P., 416(135, 136), 417(102, 109, 134, 135, 136), 418(102), 419(102, 109), *451*, *452*
Miller, M. R., 332(136), *344*
Miller, R. C., 147(88), *216*
Miller, S. L., 130(42), 131(42), *215*
Mills, G. C., 433(245), *454*
Minz, B., 254(47), *292*
Miriam, S. R., 438(285), *455*
Mirjceff, W., 371(165), *397*
Mirsky, A. E., 41(118), *70*
Mislin, H., 377(198), *398*
Mitchell, G. M., 319(89, 90), *343*
Mitchell, J. W., 410(58), *450*
Mitoma, C., 154(105), 156(105), *216*, 410(61), 412(75), *450*
Mitsuhashi, J., 269(176, 177), *295*
Mitz, M. A., 333(144), *344*
Miwa, T., 416(131), 419(111), *451*, *452*
Mizuno, D., 96(253), *115*
Mizushima, S., 84(97), 86(97), *110*
Mochnacka, I., 231(72), *242*
Möller, I., 282(254), *297*
Moir, R. J., 366(152, 152a), 367(152a), *397*
Moituda, M., 227(41), *242*
Mokrasch, L. C., 105(317), 107(317), *117*
Moldave, K., 430(221), 431(221), *454*
Mollett, J. A., 188(188), 198(188), 199 (188), *218*
Monod, J., 83(80, 85, 86), 85(122), 89 (160), 95(234), 97(257), *110*, *111*, *112*, *115*
Monroy, A., 43(127), *70*
Montgomery, J. P., 416(116), 419 (116), 420(116), 448(116), *451*
Moog, F., 2(2), 57(168), *67*, *71*
Moore, J. H., *401*
Moorefield, H. H., 439(293, 294), *455*
Moran, N. C., 409(51), *450*
Morel, F., 304(18, 20), 305(20), 308 (38), 312(38), 316(68), *340*, *341*, *342*
Morgan, J. F., 174(153), 175(153), *218*
Morgan, T. H., 260(85), *292*
Moritz, P., 385(245), *400*
Morohoshi, S., 269(167), *295*
Morris, N. R., 78(27), *108*
Morton, J. E., 378(203), 384(203), *398*
Morton, R. A., 191(197), *219*
Morton, R. K., 79(46), *109*
Moss, F., 99(270), *115*
Moudgal, N. R., 178(162), *218*
Moyed, H. S., 76(11, 12, 13), *108*
Moyer, P. W., 320(95), *343*
Müller, B., 287(293), *298*
Müller, W., 196(214), *219*
Munch-Peterson, A., 201(227), *220*
Munsick, R. A., 303(13), 305(13), 307 (35), 313(63), 316(13), *340*, *341*, *342*
Murakami, T., 447(332), *456*
Murlin, J. R., 34(88), 35(88), *69*
Murray, E. G. D., 143(75), *215*
Murray, M. R., 123(26), *214*
Murrell, W. G., 11(27), 15(41), *68*
Murty, G. G. K., 5(5), 16(5), *67*
Muth, O., 185(180), *218*
Myers, C. M., 415(127), 424(179), *451*, *453*
Myers, F. L., 386(259), *400*
Myrbäck, K., 356(86b), *394*

N

Nace, G. W., 56(164), *71*

Nachmansohn, D., 251(22, 23), *291*
Nachtigall, M., 277(229), 278(229), *296*
Nair, N. B., 384(238, 239), 387(238, 239), *400*
Naisse, J., 261(111), 262(114), 271 (114), *293*
Nakahara, H., 168(141), *217*
Nakahara, T., 15(42), *68*
Nakamura, S., 416(131), *452*
Nakano, E., 43(127), *70*
Nakao, M., 427(201), *453*
Nakayama, K., 155(111), *216*
Nakayama, T. O. M., 152(101), 206 (101), *216*
Nandi, N., 430(217), *454*
Narahara, H. T., 338(170), *345*
Narasimha Murthy, P. V., 91(195), *113*
Nason, A., 89(172), *112*
Naughton, M. A., 336(161), *345*
Neame, K. D., 351(48, 49, 50), 352 (48, 49), *393*
Needham, D. M., 227(36), *241*
Needham, J., 3(3), *67*, 444(318), 445 (321, 328), *456*
Needham, J. C., 121(1), *213*
Neidhardt, F. C., 18(48), 19(48), *68*
Neilands, J. B., 190(196), *219*
Nelson, T. C., 261(106), *293*
Nemeth, A. M., 92(201), 107(201), *113*
Neuhaus, F. C., 79(38a), *108*
Neuprez, R., 414(97), *451*
Neurath, H., 105(315), *117*, 333(147), *344*
Nevin, T. A., 196(215), *219*
Newburg, R. W., 153(103), *216*
Newey, H., 351(43), 353(60b), *393*
Nichol, C. A., 107(350), *118*
Nicholas, W. L., 188(188), 198(188), 199(188), *218*
Nicol, D. S. H. W., 336(163), 337(166), *345*
Nicol, J. A. C., 171(147), 176(147), *217*
Niegisch, A. D., 287(288), *298*
Niemierko, W., 231(63), 232(83), *242*, *243*
Nirenstein, E., 378(208), *398*
Nishi, A., 9(17), *68*
Noland, J. L., 209(252), *220*
Noll, H., 360(111), *395*
Norris, E. R., 376(191, 192), *398*
Northcote, D. H., 386(259), *400*
Northrop, J. H., 104(299), *116*, 356 (88), 357(88), 376(88), *394*
Nose, Y., 422(146), *452*
Novak, V. J. A., 261(108), 265(108), 266(108), *293*
Novelli, G. D., 146(83), 164(131), *216*, *217*
Noyes, H. M., 29(81), *69*
Nuestein, R. M., 438(286), *455*
Nunez, J. A., 282(255), *297*
Nygaard, A. P., 19(61), *69*
Nystrom, C. W., 416(108), 418(108), *451*

O

O'Brien, R., 282(257), *297*
O'Brien, R. D., 411(64), 443(313), 444 (314), *450*, *456*
Ochoa, S., 84(104), *110*
O'Connor, R., 8(14), *68*
Ofner, R. R., 438(283), *455*
Ohman, L., 47(139), *70*
Ohnishi, E., 104(302), *116*
Okada, Y. K., 260(86), *292*
Okazaki, K., 168(142), *217*
Okubo, S., 415(130), *452*
Oldfield, J. E., 185(180), *218*
Oldham, F. K., 412(76), *450*
Oomen, H. A. P. C., 354(65), 355(65), 384(65), *394*
Oparin, A. I., 128(38, 39), 131(39), *214*
Orr, A. P., 378(209), *399*
Orstrom, A., 41(115), *70*
Ostlund, E., 254(34, 35), 264(138), *291*, *294*
Otschakowskaja, S., 371(165), *397*
Ott, G. L., 207(237), *220*
Otten, C. J., 357(98), *395*
Owen, G., 380(224a), 381(224a), 384 (224a), *399*
Oxford, A. E., 364(142a), *396*

P

Paech, K., 35(91), 36(91), *69*
Paik, S. M., 254(42), *291*

Paik, W. K., 107(351), *118*
Palm, N. B., 262(116), *293*
Palmer, D. E., 92(223), *114*
Pappenheimer, A. M., 83(80), *110*, 224 (21, 22), *241*
Pardee, A. B., 73(1), 76(5, 7, 10, 16), 77(10, 17), 78(36), 80(52), 82 (75), 83(78, 79, 82), 85(123), 96 (241, 245, 250), 97(241, 257), 98 (16), 105(324, 324a), 106(324), *107*, *108*, *109*, *110*, *111*, *114*, *115*, *117*
Parke, D. V., 406(25), 407(31), 409 (47), 436(273), *449*, *450*, *455*
Parker, B. C., 88(147, 148), *112*
Parkin, E. A., 387(264), *400*
Parks, R. E., 79(48), *109*
Parnas, I., 386(260a), *400*
Parr, C. W., 81(67), *109*
Parsons, B. J., 349(27), 352(27), *392*
Parsons, D. S., 349(25), 350(25), 353 (25), *392*
Parthasarathy, D., 363(136), *396*
Pascal, R. M., 406(19), *449*
Pascual, A. A., 434(255), *454*
Passonneau, J. V., 230(62), *242*, 386 (257), *400*
Pasynskii, A. G., 128(39), 131(39), *214*
Patel, N. G., 288(300), *298*
Paterson, D. G., 444(315), *456*
Pattinson, F. L. M., 405(16), *449*
Pauly, H., 86(119), *111*
Pautard, F. G. E., 168(139), *217*
Pavan, M., 162(127), *217*, 405(8), 415 (125), *448*, *451*
Pavlov, I. P., 348(9), *392*
Pavlovskaya, T. E., 128(39), 131(39), *214*
Payne, R. W., 319(83), 320(83), *342*
Pearee, G. W., 438(284, 287, 288), 439 (296), 440(296), *455*, *456*
Pearson, O. P., 148(91), *216*
Pearson, P. B., 372(169), *397*, 426 (186), *453*
Pearson, R. M., 365(150e), *397*
Peck, B. J., 426(186), *453*
Pekarek, E., 337(168), *345*
Pelling, G., 237(106), *243*
Pentz, E. I., 137(58), 174(58), 177 (58), *215*
Perez-Gonzalez, M. D., 263(130), *294*
Perkins, E. B., 263(128), *294*
Perlmann, P., 41(116, 117), *70*
Perlzweig, W. A., 426(186), *453*
Perry, A. S., 411(72), 437(274), 439 (296), 440(296, 300, 303), 441 (300), *450*, *455*, *456*
Perry, J. J., 14(35), 18(35), *68*
Pesson, M., 195(213), *219*
Peters, R., 176(158), *218*
Petrou, W., 376(186), *398*
Petryszyn, C., 231(72), *242*
Pett, L. B., 79(39, 40), *109*
Pettinga, C. W., 333(139), *344*
Pfander, W. H., 363(135), *396*
Pfeiffer, I. W., 282(253), *297*
Pfiffner, J. J., 200(222), *219*
Pflügfelder, O., 265(142), 266(142), 278(235), 279(142), *294*, *296*
Phaff, H. J., 89(159), *112*
Phear, E. A., 99(265, 266), *115*
Phillips, J. G., 315(66), 318(77), 330 (77), *342*
Phillips, J. H., 355(65a), *394*
Phillips, P. H., 182(173), *218*
Phillipson, A. T., 362(118, 119, 123, 124, 127), 363(118, 119, 127, 131a, 133–137), 364(150c,d), 365(118, 146, 147, 148, 150), 366(118, 119), *396*, *397*
Piekering, B. T., 308(40), 312(40), *341*
Piekford, G. E., 304(21), 313(62), 315 (62, 66), 318(80), *341*, *342*
Piepho, H., 266(146, 148), 277(225), 278(225), 279(238), *294*, *296*, *297*
Pieree, H. B., 34(88), 35(88), *69*
Pieree, J. G., 304(22, 23), 309(22), *341*
Pietruszko, R., 89(174), *112*
Piez, K. A., 103(290), *116*
Pilgrim, A. F., 363(132), *396*
Pillae, V. K., 180(170), *218*
Pillai, M. K. K., 355(75), *394*
Piozzi, F., 162(127), *217*, 415(125), *451*
Pirie, A., 164(130), 189(130), *217*
Pirson, A., 179(167), 182(167), *218*
Plagge, E., 272(208), *296*
Plaut, G. W. E., 84(106), *110*
Plempel, M., 122(23), *214*

Plongeron, R., 304(25), *341*
Plumb, E. J., 357(97), *395*
Podoski, E. P., 85(127), *111*
Pollock, M. R., 19(56), *69*, 82(71), 84 (71), 86(121), *110*, *111*, 404(2), *448*
Polonovski, M., 195(213), *219*
Pon, N. G., 320(98), *343*
Pontecorvo, G., 92(213a), 94(213a), 103 (213a), *114*
Pontremoli, S., 81(68), *109*
Popenoe, E. A., 304(24), 310(54), 311 (24), *341*, *342*
Popják, G., 101(279), *115*
Porath, J., 307(27), 319(90), 320(98), *341*, *343*
Porra, R. J., 99(270), *115*
Porter, J. R., 122(5), 139(5), 174(5), 177(5), 185(5), 186(5), *213*
Porter, R. R., 358(94b), *395*
Porterfield, V. T., 437(275), 439(275), *455*
Portier, P., 388(275), 389(275), *401*
Portman, O. W., 427(193), *453*
Posner, H. S., 412(75), *450*
Possompés, B., 268(159), *294*
Postgate, J., 145(79), *215*
Postma, N., 367(156), 368(156), 370 (160), 371(156), 372(156), *397*
Potter, V. R., 78(34), 80(52), 107(337), *108*, *109*, *117*
Potts, A. M., 103(287), *115*
Powell, J. F., 5(7), 8(12), 9(7), 10 (22), 11(22), *67*, *68*
Powelson, D. M., 28(77), *69*
Power, F. W., 427(198), *453*
Powning, R. F., 388, *401*
Prange, I., 122(20), *214*
Pratt, E. M., 192(204), *219*
Prestidge, L. S., 82(75), 83(78, 79), *110*
Price, J. B., 92(200), 93(200), *113*
Prickett, C. S., 438(291), *455*
Pridham, J. B., 406(24), 416(24), 419 (117), *449*, *451*
Priest, S. G., 105(320), *117*
Prosser, C. L., 138(62), *215*, 250(17), *291*, 348(13), 356(13), 357(13), 378(13), *392*
Provasoli, L., 88(143, 144), *111*, 151 (100), 166(100), 174(149, 150), 176(149), 180(149), 200(225), 203(149, 233), *216*, *217*, *219*, *220*
Pryor, M. G. M., 234(95–97), *243*
Purvis, J. L., 319(91), *343*
Pybus, M. B., 406(19), *449*

Q

Quastel, J. H., 351(42), *393*
Quayle, J. R., 84(90), 86(90), *110*
Querol, C. G., 434(254), *454*
Quilico, A., 162(127), *217*, 415(125), *451*
Quinn, G. P., 405(12), *449*

R

Raacke, I. D., 325(117), *343*
Raben, M. S., 319(83), 320(83), *342*
Race, B., 316(68), *342*
Racokzy, A., 360(112), *395*
Radhakrishnamurty, R., 426(188), 429 (188), *453*
Radhakrishnan, A. N., 98(261), *115*
Radomski, J. L., 411(69), *450*
Radtke, A., 278(234), *296*
Raghupathy, E., 91(195), *113*, 178 (162), *218*
Rajewsky, B., 86(119), *111*
Ramachandran, J., 327(121), 328(121), *343*
Rametta, G., 283(258, 263), 284(263, 268), *297*
Ramot, B., 104(305, 306), 105(306), *116*
Ramsay, J. A., 448(335), *457*
Ranson, S. L., 80(56), *109*
Rao, D. R., 158(121), *217*
Raper, K. B., 20(62), *69*
Rapkine, L., 41(112), *70*
Rapoport, S., 104(308), 105(308), *116*
Rasmussen, H., 308(42), 312(42), *341*
Rautanen, N., 79(43), 98(43), *109*
Ravenna, C., 416(132), *452*
Ray, A. P., 406(27), *449*
Ray, D. L., 386(251), 387(251), *400*
Read, B. E., 427(194), *453*
Read, C. P., 146(85), 198(219), *216*, *219*, *401*
Reali, G., 138(63), *215*
Rebstock, M. C., 436(270), *455*

Reddy, A. R., 361(114), *395*
Reed, L. J., 199(221), *219*
Reem, G. H., 445(322), *456*
Reese, E. T., 84(115), 89(115, 158), 90(115), *111*, *112*
Reichard, P., 78(26), *108*
Reichert, E., 425(175), *453*
Reichstein, T., 288(297), *298*
Reid, E. W., 349(15, 16), 352(15, 16), *392*
Reid, M. E., 157(116), *216*
Reid, R. L., 365(150f), *397*
Reid, R. S., 363(131a), 365(146), *396*
Reitz, H. C., 412(75), *450*
Rembold, H., 288(301–303), *298*
Remington, R. E., 178(163), *218*
Remmert, L. F., 185(180), *218*
Renaud, L., 382(230), *399*
Renner, M., 288(294), *298*
Repaske, R., 85(133, 134), *111*
Ressler, C., 309(44), 310(47, 49), *341*
Reynolds, D. M., 84(113), *111*
Rich, S., 406(22), *449*
Richards, A. G., 151(99), *216*
Richert, D. A., 413(88), *451*
Richmond, V., 357(94), 358(94), *395*
Richter, C. P., 410(60), *450*
Richter, G. W., 95(233), *114*
Rieger, C., 88(138), *111*
Rifenburgh, S. A., 380(223), *399*
Rijven, A. H. G. C., 90(194), 99(263a), *113*, *115*
Riker, A. J., 125(28), *214*
Riley, M., 83(78), *110*
Rimington, C., 180(169), *218*
Rimon, A., 104(305, 306), 105(306), *116*
Ringelmann, E., 196(216), *219*
Ringer, R. L., 412(79), *450*
Rittel, W., 324(112), *343*
Rittenberg, D., 104(293, 294), *116*, 423(160), *452*
Ritter, C., 409(45), *449*
Rivers, S. L., 78(25), 102(25), *108*
Robbie, W. A., 39(101), 40(101), *70*
Robbins, P. W., 422(145), *452*
Robbins, W. E., 435(260), 440(297), 441(297), *455*, *456*
Roberts, C. W., 310(46, 49), *341*
Roberts, J. J., 89(163), *112*
Roberts, N. Q., 107(350), *118*
Roberts, R. B., 75(4), *107*
Robinson, A. E., 413(84), *450*
Robinson, D., 414(94, 95), 421(150), 428(206), 429(206), *451*, *452*, *453*
Roche, J., 283(258, 263), 284(263, 267, 268), 285(271, 274), *297*, 422(141), *452*
Rodda, H. J., 363(132), *396*
Rode, L. J., 5(8), 30(8), *67*
Roder, M., 92(222), *114*
Roeder, K. D., 355(79), 378(79), 382(79), 389(79), *394*
Roeder, M., 92(218), *114*
Rogers, S., 107(345), *117*
Romans, R. G., 332(137, 138), *344*
Romijn, C., 382(231), 389(231), *399*
Roncoli, G., 10(20), *68*
Roos, P., 319(90), 322(106), *343*
Rose, F. L., 409(48), *450*
Rose, S. M., 45(133), *70*
Rose, W. C., 154(108), *216*
Roseman, S., 419(114), *451*
Rosén, B., 381(226), *399*
Rosen, F., 107(350), *118*, 426(186), *453*
Rosenbaum, M., *401*
Rosenberg, H. R., 207(238), *220*
Rosenberger, R. F., 96(256), *115*
Roth, L. M., 287(288), *298*
Rothe, C. F., 438(284, 286), *455*
Rothman, F., 98(259), *115*
Rothstein, M., 427(196), *453*
Rotman, B., 83(84), *110*
Roush, A. H., 89(170, 171), 90(170, 171), *112*
Roussel, J. S., 440(307), *456*
Rowbury, R. G., 96(248a), *114*
Roy, A. B., 420(137), 423(149), *452*
Roy, C., 416(107), 418(107), 419(107), *451*
Royer, J., 414(97), *451*
Rubin, B., 424(167), *452*
Ruch, T. C., 122(19), 139(19), *214*
Rudinger, J., 310(51), *341*
Rueff, L., 425(175), *453*
Ruelius, H. W., 422(140), *452*
Ruge, U., 31(85), *69*
Ruiz-Amil, M., 79(49), *109*

Runnström, J., 39(102), 40(105, 107), 41(111), *70*
Rushizky, G., 83(78), *110*
Ruska, H., 251(24), *291*
Rydon, H. N., 150(97), *216*
Ryhage, R., 383(236a), 384(236a), *400*
Ryle, A. P., 334(156, 157), 336(157), *344*, 358(94b), *395*

S

Sackler, M. L., 333(140), *344*
Sacktor, B., 224(17), *241*
Sägesser, H., 279(242), 282(242), *297*
Saffran, M., 317(75), *342*
Sagan, C., 134(50), 135(51, 52), *215*
Sagara, J., 425(181), 428(181), 429(181), 429(181), *453*
Saito, M., 267(155a,b), *294*
Saito, T., 336(162), *345*
Salle, A. J., 122(6), 139(6), *213*
Salt, R. W., 141(71), *215*
Saltmarsh, M. J., 419(117), *451*
Salvatore, G., 283(263), 284(263, 268), 285(271), *297*
Salzman, N. P., 409(51), *450*
Samuels, M., 409(45), *449*
Samuelsson, B., 78(28), *108*
San Antonio, J. P., 438(279), *455*
Sanborn, R. C., 224(19), *241*
Sanchez, F. S., 434(251), *454*
Sanders, A. G., 372(170), *398*
Sandoff, H. L., 16(44), 18(44, 47), *68*
Sandrin, E., 321(104), 322(104), 327(120), 328(125), 330(125), *343*, *344*
Sanford, P. A., 353(60b), *393*
Sanger, F., 333(148, 149), 334(150–157), 336(157, 160, 161, 164), *344*, *345*
Santer, M., 144(77), *215*
Sarlet, H., 227(40), *242*, 388(274), *401*
Sarma, P. S., 91(195), *113*, 178(162), 188(187), *218*, 426(188), 429(188), *453*
Sartorelli, A. C., 78(22), *108*
Sato, T., 408(42), 423(159), *449*, *452*
Saunders, J. P., 434(249), *454*
Sawitsky, A., 202(232), *220*
Sawyer, W. H., 303(7, 13), 304(19, 21), 305(13), 307(35), 313(63), 316(13, 69), *340*, *341*, *342*
Saxena, K. N., 355(75), 383(235b), *394*, *400*
Sayers, G., 317(70), *342*
Sayers, M. A., 317(70), *342*
Sayles, L. P., 260(87), *292*
Sayre, F. W., 92(205), 93(205), *113*
Scapa, S., 444(319), *456*
Scevola, M. E., 425(174), *453*
Schabel, E., 327(121), 328(121), *343*
Schachter, D., 427(205), 428(205), 430(220), 431(205, 223), *453*, *454*
Schaefer, K., 258(59), *292*
Schäfer, W., 233(92), *243*
Schaeffer, F., 18(46), *68*
Schaffer, J. M., 424(169), *452*
Schally, A. V., 307(28), 317(75), 318(88), *341*, *342*, *343*
Scharrer, B., 258(58), 260(80, 89, 97), 261(104), 265(143), 266(143), 278(236), 279(143, 244), *292*, *293*, *294*, *297*, 314(64), *342*
Scharrer, E., 247(3), 260(80), 261(104), 265(143), 266(143), 279(143), *290*, *292*, *293*, *294*, 314(64), *342*
Scheer, B. T., 348(12), 355(71), 357(12), 378(12), *392*, *394*
Scheidy, S. F., 423(156), *452*
Schellkopf, R. L., 370(159), 371(159), *397*
Scheunert, A., 358(100, 101a), 360(101a,b), *395*
Schieblich, M., 360(101b), *395*
Schindler, O., 288(297), *298*
Schisgall, R. M., 316(69), *342*
Schlottke, E., 375(183), 376(183), *398*
Schmialek, P., 278(232, 233), 282 (232, 233), *296*
Schmid, F., 414(99), *451*
Schmidt, G., 79(44), 98(262), *109*, *115*
Schmidt-Nielsen, B., 363(128), *396*
Schmidt-Nielsen, S., 420(138), *452*
Schmiedeberg, O., 429(211), 430(211), *453*
Schmucker, T., 390(284), *401*
Schneider, D., 286(281, 282), *298*
Schneider, W. C., 431(225), *454*
Schneiderman, H. A., 10(24), 57(170),

68, 71, 222(2a), 224(9, 18, 26), 225(26, 31), 231(64–66), 238(110), *241, 242, 243,* 267(156), 277(227), 278(231, 231a,b), 280(231, 231a), 281(231b), *294, 296*
Schoffeniels, E., 352(51, 54), 353(51, 54), 373(51), *393*
Schoorl, P., 364(139a), *396*
Schopfer, W. H., 188(190), 196(214), *219*
Schonbrod, R. D., 406(29), 439(29), 440(29), *449*
Schramm, G., 357(96), *395*
Schubert, J. R., 185(180), *218*
Schulman, J. H., 351(40), *393*
Schultze, M. O., 185(179), *218*
Schwartz, E. T., 321(105), 322(105), 328(124), *343, 344*
Schweiger, A., 104(303), 107(303), *116,* 235(103), 236(103), 237(103), *243*
Schweigert, B. S., 202(231), *220*
Schwimmer, S., 383(235), *399,* 436(272), *455*
Schwink, I., 96(242), *114*
Schwyzer, R., 324(112, 113), 327(122a), 328(123), *343, 344*
Scoggins, R. B., 78(25), 102(25), *108*
Scott, D. A., 332(137), 336(158), *344*
Scudamore, H. H., 263(129), 264(129), *294*
Seaman, G. R., 89(155, 156), *112*
Sebrell, W. H., Jr., 122(18), 139(18), *214*
Sedee, J. W., 388(269), *401*
Segal, H. L., 357(97), *395*
Seifter, E., 202(232), *220*
Sekeris, C. E., 229(55), 235(55, 103a, b), 237(103a), *242, 243*
Seki, T., 429(214), 430(214), *454*
Sellers, K. C., 447(334), *457*
Senator, E., 444(317), *456*
Sendju, Y., 425(181), 428(181), 429(181, 212), 430(181, 212), *453*
Senoh, S., 429(214), 430(214), *454*
Sera, Y., 231(73), *242*
Seraidarian, K., 98(262), *115*
Sereni, E., 271(193), *295*
Sevag, M. G., 5(9), 6(9, 10), *67*
Shakespeare, N. E., 325(114), 327(114), 330(114), *343*
Shappirio, D. G., 107(338), *117,* 224(20, 23, 24, 25), *241*
Sharman, G., 366(152), *397*
Sharon, N., 162(128), *217*
Shaver, W. R., 47(137a), *70*
Shaw, T. I., 177(160), *218*
Shearer, D. A., 288(294b), *298*
Sheba, C., 104(305, 306), 105(306), *116*
Shedlovsky, T., 105(319), *117*
Sheff, M. F., 352(56), *393*
Sheldon, D. E., 34(88), 35(88), *69*
Shemin, D., 153(104), *216*
Shen, S. C., 59(177), 60(177), *71*
Shepherd, R. G., 320(95), 325(114), 327(114), 330(114), *343*
Shepherdson, M., 76(16), 98(16), *108*
Sherwin, C. P., 404(5), 406(26), 413(26), 427(197), 438(285), *448, 449, 453, 455*
Shibata, A., 416(131), *452*
Shilo, M., 85(129), 96(256), *111, 115*
Shimazono, H., 426(190), *453*
Shimp, N. F., 180(168), *218*
Shiraishi, K., 200(225), *219*
Shive, W., 157(118), 165(133), 195(133), 197(133), *216, 217*
Shizume, K., 318(79), *342*
Shore, V. G., 82(75), *110*
Shroat, J., 85(134), *111*
Shuster, L., 107(346), *117*
Silver, H., 364(144), *396*
Simmonds, S., 348(3), 356(3), *392*
Simmonet, H., 364(141), *396*
Simmons, J. E., Jr., *401*
Simms, E. S., 78(35), *108*
Simonson, D. H., 209(253), *220*
Simpson, G. G., 92(214), *114*
Sims, P., 432(236), 433(242), *454*
Sinclair, H. M., 193(208), *219*
Sinex, F. M., 154(107), *216*
Singal, S. A., 188(191), *219*
Singer, M., 253(29), *291*
Siperstein, M. D., 101(281), *115*
Sisson, S., 362(117), 367(117), *396*
Sjövall, J., 359(107), *395*
Skinner, D. M., 230(59b), *242*
Slade, H. D., 84(105), *110*

Slamp, W. C., 84(105), *110*
Sloane, N. H., 409(45), *449*
Slonimski, P. P., 19(60), *69*, 77(19a), 89(161), 90(182), 104(182), *108*, *112*, *113*
Smadel, J. E., 82(73), *110*
Smale, B. C., 410(58), *450*
Smiles, J., 364(142), *396*
Smirnov, A. J., 374(175), *398*
Smit, H., *401*
Smith, E. L., 337(167), *345*
Smith, G. C., 250(14), *291*
Smith, G. N., 413(83), *450*, *455*
Smith, H. A., 177(159), *218*
Smith, J. A. B., 365(150e), *397*
Smith, J. D., 317(76), *342*
Smith, J. N., 406(21), 407(21), 413(81, 82), 414(81, 98), 415(81, 124, 127), 418(98), 419(98), 420(98), 421(98, 124), 422(98), 423(162), 424(98, 177, 179), 428(98), 429(177, 232), 430(177), 432(177, 232, 234), 433(234), 436(21, 271), *449*, *450*, *451*, *452*, *453*, *454*
Smith, K. L., 331(131), 332(131), *344*
Smith, L. A. C., 385(244), *400*
Smith, L. F., 334(157), 336(157, 163, 164), *344*, *345*
Smith, L. H., 102(283, 283a), *115*
Smith, M. I., 438(282), *455*
Smith, N. R., 143(75), *215*
Smith, R. J., 250(12), *291*
Smyth, D. H., 349(27), 351(34, 43), 352(27, 34, 56), 353(60b,c), *392*, *393*
Smyth, R. D., 201(227, 228), 202(229), *220*
Snell, E. E., 98(261), *115*, 174(154), 180(154), 182(173), 210(254, 257), *218*, *220*
Snoke, J. E., 79(38), *108*
Snook, T., 263(128), *294*
Søndergaard, E., 122(20), *214*
Solomon, A. K., 353(58), *393*
Solomon, J. B., 92(223, 224), *114*
Sols, A., 78(31, 32), 79(31, 32, 49), *108*, *109*
Someroski, J. F., 158(121), *217*
Somers, M., 366(152, 152a), 367(152a), *397*
Sonderegger, T. B., 10(23), *68*
Sonneborn, T. M., 107(353), *118*
Soodak, M., 164(131), *217*
Sorbo, B., 434(250), 444(250), *454*
Spar, I., 56(163), *71*
Sparks, P., 372(169), *397*, 426(186), *453*
Spector, W. S., 122(17), 139(17), *214*
Spencer, B., 422(142), 423(142), *452*
Spencer, E. Y., 410(57), 411(64), *450*
Spencer, R. E. J., 10(22), 11(22), *68*
Sperber, E., 349(23), *392*
Sperber, I., 448(336), *457*
Spiegel, M., 56(166), *71*, 92(221), *114*
Spiegelman, S., 59(173), *71*, 83(84), 89(162, 179), 90(162, 179, 181), 93(179), *110*, *112*, *113*
Spillane, J. T., 438(287), *455*
Spinks, A., 409(48), *450*
Springer, F., 389(277), *401*
Springhetti, A., 279(241), *297*
Spuhler, G., 328(124), *344*
Srisuk, S., 405(13), *449*
Stack-Dunne, M. P., 320(97), *343*
Stafford, H. A., 88(135), *111*
Stahl, W. H., 287(288), *298*
Stamm, D., 286(278), *298*
Stamm-Menderez, M. D., 274(213), *296*
Standen, H., 433(246), 437(277), *454*, *455*
Stanier, R. Y., 82(77), 84(87, 88), 85(129), 88(138a), 106(88), *110*, *111*, 142(72), *215*
Stanley, R. G., 90(185), *113*
Staple, E., 78(29), 101(278), *108*, *115*
Starkey, R. L., 145(80, 81), *216*
Starks, P. B., 363(131), *396*
Staudinger, H. G., 194(209), *219*
Stearns, R. N., 92(219), 94(219), *114*
Steelman, S. L., 319(87), *342*
Steinbach, H. B., 59(173), *71*
Steinberg, D., 83(81), 93(81), *110*
Steinberg, R. A., 137(57), 186(183), *215*, *218*
Stekol, J. A., 433(238, 240), *454*
Stephens, G. C., 264(132, 134), *294*
Sternburg, J. G., 437(276), 439(293), 440(304, 305), *455*, *456*
Stetten, D., 104(300), 105(300), *116*
Stetten, D., Jr., 146(84), *216*

Stetten, R. M., 104(300), 105(300), *116*
Stevenson, I. H., 419(115), *451*
Steward, F. C., 122(10), 136(55), 139 (10), *214, 215*
Stewart, B. T., 16(45), *68*
Stewart, C. M., 413(91), *451*
Stewart, D. R., 42(124), *70*
Stewart, G. A., 331(130), *344*
Stewart, H. C., 351(40), *393*
Stierlin, H., 162(126), *217*
Stockton, J. R., 14(36), *68*
Stoeber, F., 85(124), *111*
Stohlman, E. F., 438(282), *455*
Stone, B. C., 288(294b), *298*
Stone, J. E., 78(34), *108*
Stone, R. W., 85(132), *111*
Strange, R. E., 10(18), *68*
Strittmatter, C. F., 80(58), *109*
Strohmeyer, G., 431(224), *454*
Strominger, J. L., 420(122), *451*
Strong, H., 364(143), *396*
Sturkie, P. D., 367(154), 370(154), 371 (154), 372(154), *397*
Sueoka, N., 89(176), 90(176), 99(176), *112*
Suga, T., 427(203), *453*
Sullivan, M. S., 102(283a), *115*
Sun, Y. P., 411(71), *450*
Sundaram, T. K., 426(188), 429(188), *453*
Sunde, M. L., 372(168), *397*
Sundraham, E. R. B. S., 188(187), *218*
Sussman, M., 23(70), 24(70), 25(74), 26(74), *69*, 163(129), *217*
Sutliffe, J. F., 136(55), *215*
Suzaki, T., 447(332), *456*
Suzuki, T., 227(41), *242*, 408(42), 423 (159), *449, 452*
Swan, J. M., 310(49), *341*
Swann, M. M., 40(108), *70*
Swartz, M. N., 104(310), 105(310), *116*
Sweeney, T. R., 438(281), *455*
Swenson, P. A., 82(76), *110*
Sydenstricker, V. P., 188(191), *219*
Sykes, W. O., 423(157), *452*
Sylvester, N. R., 160(124), *217*
Synge, R. L. M., 128(39), 131(39), *214*, 362(126), 363(126), 365(126), *396*
Syrett, P. J., 84(91), 86(91), 104(91), *110*
Szulmajster, J., 18(46), *68*

T

Tabone, D., 416(105), 418(105, 106), 419(118), *451*
Tabone, J., 416(105), 418(105, 106), 419(118), *451*
Taggart, J. V., 427(205), 428(205), 430 (220), 431(205, 223), *453, 454*
Tahori, A. S., 440(306), *456*
Takahashi, M., 444(320), 446(329), *456*
Takamatsu, M., 425(181), 427(195), 428(181), 429(181), 430(181), *453*
Takano, K., 419(111), *451*
Takemori, A. E., 107(347), *118*
Talalay, P., 48(143), *71*
Talbot, F. H., 380(222), *399*
Talwar, C. P., 92(197), 94(197), *113*
Tam, N.-D., 287(287), *298*
Tamura, G., 206(236), *220*
Tanaka, M., 232(79), *243*
Tang, J., 357(94, 94a), 358(94, 94a), *395*
Tang, P. S., 57(169), *71*, 90(193), *113*
Tang, Y. W., 31(83), *69*
Tannenbaum, S., 107(335), *117*
Tarr, H. L. A., 10(21), *68*
Tarver, H., 93(225, 226), *114*
Tata, J. R., 92(215), *114*
Tatum, E. L., 89(173), *112*
Taub, R., 252(25–28), *291*
Tavormina, P. A., 201(226), *219*
Taylor, A. C., 125(29), *214*
Taylor, C. B., 349(27), 351(34), 352 (27, 34), *392, 393*
Taylor, D. J., 406(27), *449*
Taylor, H. F., 405(18), 406(19), *449*
Taylor, N. B., 348(8), 357(8), *392*
Taylor, W. H., 356(93a), 357(91–93a), 358(91), 359(91), *395*
Telfer, W. H., 229(57, 59), 230(59a), *242*
Templeton, W. H., 438(289), *455*
Ten Broeck, C., 105(318), *117*
Ten Cate, J., 254(45), *291*

Terriere, L. C., 406(29), 407(37), 432 (235), 433(235), 439(29), 440 (29), *449, 454*
Thannhauser, S. J., 98(262), *115*
Thimann, K. V., 138(61), *215*
Thomas, L. M., 285(272), *297*
Thomassey, S., 416(105), 418(105), *451*
Thompson, C. C., 90(184), *113*
Thompson, E. O. P., 334(153, 154, 155), *344*
Thompson, R. C., 31(84), *69*
Thompson, T. A., 328(124), *344*
Thomsen, E., 279(240, 243), 282(250, 251, 254), *297*
Thomsen, M., 270(189), *295*
Thorn, N. A., 302(5), *340*
Thorpe, W. V., 422(144), 423(163), 442(144, 312), *452, 456*
Timasheff, S. N., 333(142), *344*
Tinbergen, L., 374(177), *398*
Tinellii, R., 13(30), *68*
Tobita, T., 104(312), *116*
Tobler, L., 359(103), *395*
Todd, A. R., 415(129), *452*
Tolbert, N. E., 90(191), 91(191), 104 (191), *113*, 416(108), 418(108), *451*
Tomchick, R., 426(189), *453*
Toohey, J. I., 201(227, 228), *220*
Torriani, A., 79(41), 96(41), 98(258, 259), *109, 115*
Totani, G., 427(202), *453*
Towers, G. H. N., 416(107), 417(133), 418(103, 107), 419(107), *451, 452*
Tracey, M. V., 386(260), *400*
Trager, W., 122(3, 14), 139(14), 176 (156), 208(14), *213, 214, 218*
Treccani, V., 407(40), *449*
Treherne, J. E., 355(73, 77), 356(78), *394*
Tripett, S., 309(44), *341*
Tristram, H., 86(120), *111*
Trucco, R. E., 357(94, 94a), 358(94, 94a), *395*
Truesdail, J. H., 170(146), *217*
Tsao, T.-C., 338(172), *345*
Tscherniak, A., 372(167), *397*
Tschernikov, A. M., 406(28), *449*
Tsukamoto, M., 440(301), 441(301), *456*
Tsunoo, S., 447(332), *456*
Tsuyoshi, I., 424(166), *452*
Tsuyuki, H., 448(337), *457*
Tucker, R. G., 85(131), *111*
Tuppy, H., 309(45), 334(151, 152), *341, 344*
Turner, J. F., 80(60, 61), 81(61), *109*
Turner, R. A., 304(23), *341*
Turner, R. S., 257(55), *292*
Tuttle, L. C., 10(23), *68*
Twarog, B. M., 255(36), 256(36), *291*
Tyler, A., 39(103), *70*
Tyler, D. B., 80(53,54), *109*
Tyrsarowski, W., 19(60), *69*

U

Udaka, S., 77(17a), *108*
Udenfriend, S., 103(291), *116*, 154 (105), 156(105), *216*, 410(61), 412(75), *450*
Ullmann, T., 383(234), *399*
Umbarger, H. E., 76(6, 8, 9, 15), 95 (236), 96(9, 249), 105(236), *108, 114*
Underhill, F. P., 409(49), *450*
Underwood, E. J., 182(175), 184(175), *218*
Unger, H., 268(165a), 269(185–187), 270(187, 190a), *295*
Uranga, J., 304(19), *340*
Urbani, E., 59(175), *71*
Ussing, H. H., 304(17), *340*
Uyeda, K., 429(233), 432(233), *454*

V

Vallee, B. L., 179(166), *218*
Vandel, A., 283(259), *297*
van den Broek, C. J. H., 359(106), *395*
van den Driessche, J., 376(189), *398*
van den Oord, A. H. A., 383(236a), 384 (236a), *400*
Van der Kloot, W., 268(166), *295*
Van der Scheer, J., 320(95), *343*
van Dyke, H. B., 302(3), 303(3, 13), 305(13), 307(34, 35), 313(63), 316(13), *340, 341, 342*

van Herwerden, M., 377(195), *398*
Van Slyke, D. D., 154(107), *216,* 377(194), *398*
van Niel, C. B., 142(73), 143(74), *215*
VanderWende, C., 180(168), *218*
van Trigt, H., 380(218), *399*
van Wagtendonk, W. J., 208(249), 209(253), *220*
van Weel, P. B., 355(70), 380(219), 381(219, 227), 383(235c), 384(239c), 385(235c, 241, 242, 243, 244), *394, 399, 400*
Varela, G., 434(253), *454*
Varner, J. E., 107(330), *117*
Varrone, S., 283(263), 284(263), *297*
Vaughan, M., 83(81), 93(81), *110*
Vegotsky, A., 30(82), *69*
Velasco, J., 38(95), *69,* 107(331), *117*
Velluz, L., 310(52), *341*
Verron, H., 288(293a), *298*
Verzár, F., 349(14), 350(14), 351(14), 353(14), *392*
Viehoever, A., 192(203), *219*
Vincent, D., 250(11, 16), *291*
Vincent, T. N., 349(26), 351(26), *392*
Vinter, V., 14(34), *68*
Visana, A. M., 357(*see* footnote), *401*
Vishniac, W., 144(77), *215*
Visscher, M. B., 352(55), *393*
Vogel, B., 147(86), *216*
Vogel, H. J., 74(2), 95(237, 237a), 96(251), 97(237), 98(260), 105(251), *107, 114, 115,* 123(25), *214*
Vogell, W., 230(60), *242*
Vogt, H., 349(21), *392*
Vohra, P., 155(110), *216*
Volcani, B. E., 201(227, 228), 202(229), *220*
Volini, M., 333(144), *344*
Volkmann, C. M., 180(171), *218*
von Bertalanffy, L., 126(31), *214*
von Buddenbrock, W., 378(207), 382(207), *398*
von Fürth, O., 378(216), *399*
Vonk, H. J., 348(4), 351(37), 356(84, 85), 357(4, 85), 358(4), 359(4, 105), 360(4, 84), 361(115), 367(156), 368(156), 370(160), 371(156), 372(115, 156), 374(105), 375(115), 376(105, 115), 377(105), 378(202, 204, 210), 380(224), 381(202, 204), 382(202, 204, 210), 383(202, 204, 210, 236), 384(202, 210, 236), 385(202, 210), *392, 393, 394, 395, 396, 397, 398, 399, 400*
von Kolnitz, H., 178(163), *218*
von Möllendorff, W., 381(229), *399*
von Sallmann, L., 157(116), *216*
von Swiecicki, H., 373(174), *398*
Voss, G., 383(235a), *399*

W

Wagenknecht, C., 104(308), 105(308), *116*
Wagner, R. P., 77(19), *108*
Wain, R. L., 405(9, 17, 18), 406(19), *448, 449*
Wainio, W. W., 180(168), *218*
Wainwright, S. D., 89(175), 90(175), *112*
Waisman, H. A., 103(288, 289, 292), *116*
Wald, G., 107(332), *117,* 132(45), *215,* 254(33), *291*
Waldschmitt, M., 200(223), *219*
Walker, D. A., 29(80), 36(93), 37(93), 38(93), *69*
Walker, D. W., 80(56), *109*
Walker, J. B., 100(273, 274, 275), 101(273, 274, 275), *115*
Walker, N., 407(38, 39), 408(39), *449*
Waller, J. P., 310(50), 327(119, 120), 328(127), 330(126), *341, 343, 344*
Walpole, A. L., 407(34), *449*
Walsh, J. T., 287(286a), *298*
Walter, C. F., 78(33), *108*
Walter, W. G., 371(164), *397*
Walters, C. P., 48(145), 49(145), 50(147), *71*
Wang, C. H., 440(299), 441(299), *456*
Warburg, O., 38(96), 39(104), *69, 70*
Wardlaw, A. C., 338(171), *345*
Waring, H., 302(2), 303(8, 14), 318(78), *340, 342,* 366(152, 152a), 367(152a), *397*
Warth, A. H., 388(271), *401*
Wasdell, M. R., 423(163), *452*
Waterhouse, D. F., 378(213), 382

(231b), 383(213), 385(213), 388 (213, 266), *399*, *401*
Watson, M. R., 160(124), *217*
Waugh, D. F., 333(140), *344*
Webb, W. L., 364(145), *396*
Weber, G., 107(341, 348), *117*, *118*
Weber, H., 221(2), *241*
Weber, R., 59(174), *71*
Wehrmeister, H., 389(278), *401*
Weiden, M. H. J., 425(171, 172), *453*
Weikel, J. H., 438(290), *455*
Weil-Malherbe, H., 78(30), 79(30), *108*
Weinstein, M. J., 278(231b), 281 (231b), *296*
Weirich, G., 279(247), 282(247), *297*
Weiss, P., 125(29), *214*
Weissbach, H., 154(105), 156(105), 201(227, 228), 202(229), *216*, *220*
Weissburger, E. K., 407(35), *449*
Weissburger, J. H., 407(35), *449*
Weller, R. A., 363(132), *396*
Welsh, J. H., 247(1, 2), 249(7), 250 (15), 251(2), 252(25–28), 253 (2), 255(51), 256(54), *290*, *291*, *292*
Wender, S. H., 416(108), 418(108), *451*
Wense, T., 254(32), *291*
Wenzel, M., 274(213b), *296*
Wertheim, G., 198(219), *219*
Wertheimer, E., 349(20), *392*
Westenbrink, H. G. K., 350(30), *393*
Westerfeld, W. W., 413(88), *451*
Westphal, O., 162(126), *217*
Westphal, U., 105(320), *117*
Weygand, F., 200(223), *219*
Wharton, D. R. A., 287(285, 286, 286a), *298*
Wharton, M. L., 287(285, 286, 286a), *298*
Whitaker, D. M., 38(99), *70*
White, G. F., 377(194), *398*
White, G. J., 23(70), 24(70), *69*
White, K., 422(144), 442(144, 312), *452*, *456*
White, W. C., 438(281), *455*
White, W. F., 320(96), 325(115), 328 (126), 330(127), *343*, *344*
Whitehouse, M. W., 78(29), 101(278), *108*, *115*
Whiteley, A. H., 42(125), *70*
Wiame, J. M., 96(243), *114*
Wigglesworth, V. B., 147(89), *216*, 221(1), 226(33), 230(61a), *241*, *242*, 265(140), 266(140, 149, 150), 272(211), 275(211), 276(211), 277(150, 228), 278(150), 279 (150), 282(248, 248a), *294*, *296*, *297*, 378(211), 382(211), 387 (211), 388(211), 389(211), *399*
Wightman, F., 405(18), 406(19), *449*
Wijesundera, S., 95(235), 96(248), *114*
Wilbrandt, W., 350(31), *393*, *401*
Wilbur, K. M., 168(140), *217*
Wilhelmi, A. E., 304(21), *341*
Wilhelmson, D. F., 333(140), *344*
Williams, C. M., 107(338), *117*, 224(9, 18–22, 24–30), 225(26–30), 229 (57), 230(59a, 62), 231(64, 66), *241*, *242*, 266(151, 152), 272(152, 212), 275(212, 219), 277(226), 278(230), *294*, *296*, 386(257), *400*
Williams, M. H. C., 407(34), *449*
Williams, R. H., 338(170), *345*
Williams, R. J., 165(133), 170(145, 146), 195(133), 197(133), *217*
Williams, R. T., 403(1), 404(1), 405 (1), 406(1, 25), 407(1), 409(1, 47), 410(1, 59), 413(1), 414(1, 94, 95), 420(1), 421(150), 423(1, 162), 425(1), 426(1), 427(1), 428 (206), 429(206), 432(1), 433(1), 435(1), 436(1, 273), 437(1), *448*, *449*, *450*, *451*, *452*, *453*, *455*
Williams-Ashman, N. G., 48(143), *71*
Willier, B. H., 380(223), *399*
Willson, S. D., 325(114), 327(114), 330 (114), *343*
Wilson, A. C., 77(17), *108*
Wilson, B. W., 89(149a), *112*
Wilson, E. O., 287(291, 292), *298*
Wilson, J. B., 251(23), *291*
Wilson, P. W., 85(132, 133), *111*
Wilson, R. M., 201(227), *220*
Wilson, T. H., 349(14a, 26), 351(26), 353(14a), *392*
Wilt, F. H., 107(352, 354), *118*

Wiltshire, G. H., 407(38, 39), 408(39), *449*
Winnek, P. S., 177(159), *218*
Winteringham, F. P. W., 438(289), *455*
Wiseman, G., 351(44–50), 352(48, 49), *393*
Wisnicky, W., 207(237), *220*
Wisseman, C. L., 82(73), *110*
Witt, J. M., 439(292), 440(292), *455*
Wlodavez, P., 232(83), *243*
Wojtczak, A., 232(83), *243*
Wolf, J. T., 438(285), *455*
Wolf, S., 356(82), 357(94, 94a), 358 (94, 94a), *394*, *395*
Wolfe, M., 183(177), *218*
Wolff, H. G., 356(82), *394*
Wolvekamp, H. P., 356(84), 360(84), 373(172), 374(177, 178), *394*, *398*
Wood, J. L., 433(245), *454*
Wood, P. B., 422(144), 433(239), 442 (144, 312), *452*, *454*, *456*
Woodard, G., 438(278), *455*
Woodbury, L. A., 317(70), *342*
Woodcock, D., 405(14, 15), 409(15, 43), *449*
Woods, D. D., 95(235), 96(248, 248a), *114*
Woolley, D. W., 191(198), 211(258), *219*, *220*
Woolner, M. E., 328(124), *344*
Wootton, I. P., 431(228), *454*
Work, E., 155(112), *216*
Wormser, E. H., 76(10), 77(10), *108*
Worrell, C. S., 413(83), 436(271), *450*, *455*
Wright, B. E., 14(38), 21(64), 22(64, 67), 23(67), 24(38, 71, 73), 25 (38, 64, 73), 26(38, 64), 27(64, 76), 28(71, 73, 79), 29(79), 30 (79), 67(184), *68*, *69*, *71*, 104 (295), 105(295), *116*
Wright, D. R., 372(170), *398*
Wright, L. D., 201(226), *219*
Wu, H. Y., 90(193), *113*
Wu, P. H. L., 158(121), *217*
WuChang, M. L., 427(204), 428(204), 429(204), *453*
Wugmeister, M., 228(53), *242*
Wurster, D. H., 332(136), *344*
Wyatt, G. R., 140(68), 141(68), *215*, 227(37), 231(37, 67, 70, 71), 232 (81), *241*, *242*, *243*
Wyatt, S. S., 227(37), 231(37), *241*
Wyckoff, L. B., 103(289), *115*
Wyngaarden, J. B., 78(21), *108*
Wynne, A. M., 79(39, 40), *109*
Wyss, O. J., 14(36), *68*

Y

Yajima, H., 321(105), 322(105), 327 (122), 328(122, 124), 330(122), *343*, *344*
Yamada, M., 408(42), *449*
Yamaha, T., 416(121), 419(110, 121), *451*
Yamamoto, R. T., 287(286b), *298*
Yamasaki, M., 93(227), *114*
Yanagisawa, I., 427(201), *453*
Yanagisawa, T., 61(181), *71*
Yanaihara, N., 327(122), 328(122), 330(122), *344*
Yanari, S., 79(38), *108*
Yanofsky, C., 96(247), *114*
Yarwood, E. A., 188(188), 198(188), 199(188), *218*
Yates, R. A., 76(5), 96(241), 97(241), *107*, *114*
Yčas, M., 90(183), *113*
Yocum, C. S., 142(73), *215*
Yokoyama, H., 152(101), 206(101), *216*
Yonge, C. M., 355(67, 68, 69), 361(68), 378(199, 200), 380(67, 199, 200), 381(199), 384(199, 200, 239d), *394*, *398*, *400*
Yoshida, A., 93(227), 104(312), *114*, *116*
Yoshida, M., 260(94), *293*
Yoshida, T., 207(243), *220*
Yoshikawa, H., 423(159), 427(201), *452*, *453*
Young, E. G., 137(58), 174(58), 177 (58), *215*
Young, I. E., 18(50, 51), 19(50), *68*
Young, J. L., 107(330), *117*
Young, L., 407(36), 433(237, 244), *449*, *454*
Young, Z. I., 271(192), *295*
Yura, T., 98(260), *115*

Z

Zak, G., 254(43), *291*
Zaki, F. G., 384(239c), *400*
Zaoral, M., 310(51), *341*
Zarrow, I. G., 317(74), *342*
Zarrow, M. X., 317(72, 74), *342*
Zeid, M. M. I., 435(259), *455*
Zerahn, K., 304(17), *340*
Ziegler-Günder, J., 268(163), *294*
Zielinski, M. A., 41(114), *70*
Zavarzin, G. A., 183(178), *218*
Zeledon, R., 198(219), *219*
Zeuthen, E., 148(92), *216*
Zillican, F., 191(199), *219*
Zillig, W., 235(102), *243*
Zimmerman, S. B., 107(344), *117*
ZoBell, C. E., 146(83), *216*
Zuber, H., 324(112), *343*
Zuelzer, W. W., 445(327), *456*
Zukel, J. W., 406(30), *449*
Zwicky, K., 226(33), *241*

SUBJECT INDEX

A

Abomasum,
  fatty acids and, 363, 364
  secretions of, 361–362, 364
Absorption,
  intestinal,
    intestinal movement and, 356
    polarity of, 349
  particle size and, 378
Acanthamoeba castellanii, pyrimidine requirement of, 166
Acantharia, skeleton of, 168
Acetaldehyde,
  acetoin formation from, 171
  threonine synthesis from, 151
p-Acetamidobenzoic acid, excretion of, 423
2-Acetamidofluorene,
  detoxification of, 407
  tumors and, 407
Acetanilide,
  detoxification of, 446
  excretion of, 423
Acetate,
  abiogenic formation of, 131
  bacterial morphology and, 145
  cholesterol synthesis from, 101
  enzyme induction and, 89
  formation, carbon dioxide and, 144
  morphogenesis and, 27
  ruminant and, 362, 365
  spore germination and, 5
  sporulation and, 13, 18
  utilization, 147, 362
    ethanol and, 89
Acethion, toxicity of, 444
Acetobacter suboxydans, amino acid requirement of, 159
Acetoin, nonenzymic formation of, 171
Acetolactate, valine synthesis and, 152
Acetone, bacterial morphology and, 145
N-Acetylamino acid acylase, fluorophosphonates and, 436
Acetylation, detoxification and, 423–425, 447
Acetylcholine,
  activity of, 253
  biosynthesis, accumulation and axonal transport of, 251
  cell membrane and, 204
  distribution of, 249–251
  invertebrates and, 248–253
  myotropic activity and, 269, 270
  site and mode of action, 251–253
  triethyl analog of, 252
  tropic action of, 253
Acetylcholinesterase, induction of, 92
Acetyl coenzyme A,
  acetylations and, 425
  leucine synthesis and, 152
  threonine and, 152
  valine synthesis and, 152
N-Acetylcysteine, detoxification and, 432
N-Acetyldopamine, tanning and, 235–236
Acetylgladiolic acid, fungistatic activity of, 435
N-Acetylglutamate,
  synthesis, inhibition of, 77
N-Acetyl-4-hydroxytyramine, detoxification of, 415
Acetyl indoxyl, oxidation of, 412
Acetylisatin, formation of, 412
Acetylserine, α-melanocyte-stimulating hormone and, 321, 328
Acetyl transfer, carnitine and, 205
Acetyl tridecapeptide, melanotropic activity of, 329
N-Acetyltyramine, tanning and, 235
Achatina,
  diet, enzyme production and, 385
Achromobacter,
  cytochrome a_2, induction of, 86
Achromobacteriaceae, enzyme induction in, 84, 86
Achromotrichia, copper and, 181
Aconitase, iron and, 179
Actidione, nitrate reductase formation and, 91
Actinomyces oligocarbophilus, 145

Actinomycetes,
  enzyme induction in, 84, 87
  lysine biosynthesis by, 123–124
Actinomycin I, prolines and, 154
Actinozoans, N-methyl pyridinium hydroxide in, 426
Active transport, vitamin B_6 and, 198
Actomyosin, calcium and, 175
Acyl groups,
  transfer, vitamins and, 198–199
Adenase, induction of, 89, 90
Adenine,
  assay of, 165
  feedback inhibition by, 78
  folic acid and, 199
  pseudocobalamin and, 164
  requirement for, 165–167
  vitamin B_{12} and, 201
Adenosine, spore germination and, 8
Adenosine deaminase,
  induction of, 92
  spore germination and, 8
Adenosine diphosphate, 164
  energy-supplying pathways and, 79
  Pasteur effect and, 80
  product inhibition by, 78, 79
  spore germination and, 18
Adenosine-monophosphate, 164
  histidine synthesis and, 155–156
  sea urchin eggs and, 41, 42
Adenosine-3′-phosphate-5′-phosphosulfate, sulfate activation and, 145
Adenosine-5′-phosphosulfate, sulfate activation and, 145
Adenosine ribosidase, spores and, 17
Adenosine triphosphatase,
  amphibian differentiation and, 58, 61
  calcium and, 175
  seed germination and, 37–38
Adenosine triphosphate, 164
  production, rate of, 79
  requirement for, 196
  sea urchin eggs and, 41, 42
  spore germination and, 9, 18
S-Adenosylmethionine, vitamin B_{12} and, 153
Adenylcobamide, structure of, 201
Adiantum macrophyllum, glycosides in, 416
Adipose tissue, insulin and, 338
Adrenal gland,
  ascorbic acid, corticotropin and, 317
  hormones of, 300
  melanocyte-stimulating hormone and, 317
Adrenaline, *see also* Epinephrine
  deactivation of, 426
  invertebrates and, 253–255
  myotropic activity and, 269
Adrenocorticotropic hormone, *see* Corticotropin
Adsorption, polymerization and, 132
Aedes aegypti,
  amino acid requirement of, 161
  benzoic acid and, 429
  calcium and, 175
  carnitine and, 205
  DDT metabolism in, 440
  glycine and, 432
  growth, nucleic acid and, 167
  magnesium requirement of, 176
  sodium and, 174
Aedes concolor,
  iron requirement of, 179
  sodium requirement of, 174
Aerobacter aerogenes,
  citrate permease of, 85
  enzyme repression in, 95, 96
  feedback inhibition in, 77
  glyceral dehydrogenase, induction of, 86
  iron requirement of, 179
  methionine synthesis by, 153
Age, spore germination and, 5
Alabama argillacea, zinc requirement of, 178
Alanine,
  formation of, 150
    abiogenic, 131
  insulin and, 336
  β-melanocyte-stimulating hormone and, 323
  pupal molt and, 227, 228, 229
  requirement for, 150, 159, 160
  spore germination and, 5–9, 15, 16, 65
  transport of, 351, 352
β-Alanine,
  abiogenic formation of, 131
  pantothenate and, 198
  pupa and, 228

Alanine-*α*-ketoglutaric acid transaminase, differentiation and, 29–30
Alanine racemase, spores and, 16–17
Albinism, tyrosine and, 156
Albumin,
  egg,
    amino acid requirement and, 159
    pepsin and, 357–358
  spore germination and, 9
Albumin gland, sex hormone and, 271
Alcohol(s),
  detoxification of, 419
  β-glucuronidase and, 419
  secondary, detoxification and, 405
Alcohol dehydrogenase(s),
  metamorphosis and, 226
  zinc and, 178
Alcyonidium verrilli, juvenile hormone in, 280
Alcyonium carneum, juvenile hormone in, 280
Aldehydes, polyglycine and, 132
Aldehyde oxidase, molybdenum and, 183
Aldolase,
  cobalt and, 184
  spores and, 15
Aldrin, oxidation of, 411
Algae,
  blue green, sodium and, 137, 173–174
  boron deficiency in, 186
  bromide and, 177
  calcium requirement of, 175
  carotenoids, vitamin A and, 206
  cobalt and, 184
  copper requirement of, 180
  enzyme induction in, 88–89
  enzyme repression in, 98
  iron and, 179
  marine, requirements of, 203
  sterols and, 208
  zinc and, 178
Alginase, fish and, 376
Alimentary canal, reduction of, 389
Aliphatic compounds, detoxification of, 404–406
Alkaloids, tryptophan and, 157
Alkaptonuria, tyrosine and, 156–157
Alkylarsonic acids, methylation of, 426
Alligator,
  aromatic acids and, 430, 432
  demethylation by, 411
Allothreonine, 151
Alopecia, copper and, 181
Aluminum, cell growth and, 186
Amanita muscaria, vanadium in, 186
Amblystoma punctatum,
  gastrula, respiration of, 52–53
Amebocytes, enzyme potency of, 384
Ameirus,
  gut, bacteria in, 376
Amidase(s), detoxification and, 435–436
Amine(s),
  acetylation of, 423–425
  detoxification of, 419
Aminoacetonitrile, polymerization of, 131–132
Amino acid(s),
  abiotic polymerization of, 131–132
  free, cocoon spinning and, 227–228
  interconversions, vitamin B_6 and, 197
  intestinal absorption of, 351–352, 355
  metabolism, metamorphosis and, 227–231
  morphogenesis and, 27
  origin of, 130–131
  osmotic balance and, 138–140
  permeases, induction of, 85
  pool, sporulation and, 14
  pseudoplasmodium formation and, 21–23
  puparium formation and, 229
  requirement for, 158–159, 212–213
  sea urchin egg development and, 41, 42
  seed germination and, 35–36
  spore germination and, 5, 9
  transamination, nonenzymic, 171
D-Amino acid(s), utilization of, 152
m-Aminobenzoic acid, detoxification of, 442
o-Aminobenzoic acid, detoxification of, 416
p-Aminobenzoic acid,
  acetylation of, 205, 423–425
  biosynthesis of, 201
  detoxification of, 414, 429
  folic acid synthesis and, 200
  function of, 201
  microorganisms and, 170
  requirement for, 201

sulfate and, 420, 421
α-Aminobutyrate,
  abiogenic formation of, 131
  methionine synthesis from, 153
  threonine requirement and, 151, 152
γ-Aminobutyrate,
  carnitine and, 205
  glutamate and, 150
γ-Aminobutyrate transaminase, induction of, 89
α-Amino-β-ketoadipic acid, porphyrins and, 150
2-Amino-1(6)-naphthol, formation of, 407
Aminonitrocresol,
  acetylation of, 424
  detoxification of, 415
Aminopeptidase(s), magnesium and, 176
Aminophenol(s),
  acetylation of, 423
  aniline and, 407, 409
m-Aminophenol, detoxification of, 415, 421
o-Aminophenol, detoxification of, 420
Aminopolysaccharides, formation of, 163
Aminopropanol, threonine and, 152
Aminopyrine, detoxification of, 446
p-Aminosalicylic acid, glutamine and, 427, 431
Amino sugars, insect hemolymph and, 140
Ammocoetes, thyroid hormone in, 285
Ammonia,
  aminoacetonitrile formation and, 132
  amino acid formation and, 130, 131
  arginine-vasopressin and, 311
  oxytocin and, 309
  oxidation of, 144
  primitive biosphere and, 130
  proteolysis and, 14
  sea urchin egg development and, 41, 42
  seed germination and, 36
  ruminants and, 362, 364–365
  spore germination and, 7, 8
Ammonium chloride, volcanic gas and, 130
Ammonotelism, metamorphosis and, 61
Amoeba, food intake by, 378
Amphetamine, detoxification of, 446
Amphibia,
  acetylation in, 424
  aromatic acids and, 429, 431
  blood osmotic pressure of, 139
  differentiation,
    altered enzymes and, 57–63
    energy requirement for, 52–57
  digestion in, 373–374
  ethereal sulfates in, 420, 421
  glucuronides in, 414
  neurohypophyseal hormone activity in, 303–304
  tapetum lucidum of, 164
  tocopherol and, 192
  venom, serotonin in, 157–158
  vitamin A in, 206
Amphioxus,
  phagocytosis in, 381
  thyroid hormone in, 285
Amylase(s),
  amphibian differentiation and, 58
  bile and, 372
  chloride and, 171, 177
  crystalline style and, 384
  ethionine and, 93
  frog pancreas and, 374
  halides and, 124
  inactive form of, 104–105
  loss of, 389
  reptile, pH optimum of, 373
  salivary, 357
    frog and, 374
  secretion,
    fasting and, 364
    invertebrates and, 382
    starch and, 385
  seed germination and, 32
  temperature and, 361
α-Amylase, occurrence of, 383
β-Amylase, food and, 383
n-Amyltrimethylammonium ions, acetylcholine and, 252, 253
Anabaena cylindrica,
  calcium requirement of, 175
  molybdenum and, 183
  sodium and, 173–174
Anaerobes,
  iron and, 179
  sporulation of, 13

Anaerobiosis, amino acid absorption and, 351
Anatopynia, enzyme repression in, 99
Androgenic gland,
  function of, 271–272
  hormone of, 262
Anemia,
  copper and, 182
  folic acid and, 200
  vitamin B_{12} and, 200, 203
Angiosperms, enzyme induction in, 90
Anguis,
  aromatic acids and, 429
  ethereal sulfates in, 421
Aniline,
  acetylation of, 423
  oxidation of, 407, 409
Animal(s)
  amino acid requirements of, 158–159
  carbon balance and, 126–127
  evolution, nutritional requirements and, 212
  fluid medium maintenance in, 138–141
  land, vitamin A of, 206
  marine, internal pH of, 138
  minimal possible size of, 148
  osmotically active substances in, 212
  sterols of, 208
Animal protein factor, *see* Vitamin B_{12}
Annelids,
  acetylcholine in, 249
  adrenaline in, 254
  hepatopancreas and, 381
  juvenile hormone in, 280
  Pasteur effect in, 80
  polychaete, neurosecretion in, 258–259
Anodonta,
  heart, adrenaline and, 254
  5-hydroxytryptamine in, 255
Anopheles,
  calcium requirement of, 175
  potassium and, 174
Anopla, juvenile hormone in, 280
Anostracans, enzyme repression in, 99
Anserine, histidine and, 156
Ant(s), pheromones of, 287
Antherea pernyi, phosphorus in, 232
Anthonomus grandis, DDT metabolism in, 440
Anthozoa, juvenile hormone in, 280
Anthracene, detoxification of, 433
Anthranilic acid,
  detoxification of, 418, 429, 430
  tryptophan synthesis and, 157
Anthremus muscorum, silk digestion by, 388
Antibiotics,
  chitinase production and, 386
  synthesis, feedback and, 106
Antibody,
  carbamyl phosphate synthetase and, 62
  induced enzymes and, 94
  zymogens and, 105
Antidiuretic activity,
  batrachians and, 304
  chick and, 303
  mammals and, 303, 305, 316
Antigens,
  amphibian differentiation and, 56
  sea urchin egg development and, 41
Anti-gizzard-erosion factor, nature of, 211
Aphanacin, vitamin A and, 206
Aphanin, vitamin A and, 206
Aphins, detoxification of, 415
Aphis, glucosides in, 415
Apis mellifera,
  adrenaline in, 254
  juvenile hormone in, 280
Aplausobranchiata, vanadium in, 185
Aplysia punctata, acetylcholine effect on, 250
Appendices pyloricae, structure and function of, 375–376
Arabinose,
  glycoside formation and, 418
  intestinal absorption of, 349–350, 354
Arachidonic acid,
  activity of, 193
  synthesis of, 194, 197
Arachnida,
  acetylcholine and, 249
  endocrine glands in, 271
  juvenile hormone in, 280
  neurosecretion in, 261, 262
Arachnoidea, hepatopancreas in, 381
Arbacia,
  eggs,
    glycolytic enzymes in, 47

    permeability of, 42, 66
    pyridine nucleotides in, 47
Arabacia punctulata, juvenile hormone and, 281
Arbutin, detoxification of, 406
Arginase,
  amphibian differentiation and, 61
  chick embryo development and, 51
  enzyme repression and, 100
  inactive form of, 104
  induction of, 92
  manganese and, 182
  stimulation and inhibition of, 51, 66
Arginine,
  arginine-vasopressin and, 310–311
  arginine-vasotocin and, 312
  diapause and, 228
  feathers and, 149
  glutamate and, 150
  β-melanocyte-stimulating hormone and, 319, 320, 323, 324
  metabolic role of, 154–155
  requirement, 154, 159–163
    creatine and, 101, 159
  sclerotization and, 234
  synthesis,
    aspartate and, 150
    feedback inhibition of, 77
    rate of, 154
    repression of, 95, 97, 98
Arginine-oxytocin, *see also* Arginine-vasotocin
Arginine-oxytocin,
  bird, 307
  fish, 308
  frog, 308
Arginine-vasopressin,
  activities, species and, 305
  antidiuretic effect, species and, 316
  chemical synthesis of, 311
  occurrence of, 307
  structure of, 310–311
Arginine-vasotocin,
  activities, species and, 305, 315
  antidiuretic effect, species and, 316
  bird, 307, 312
  chemical synthesis of, 313
  function of, 301
  structure of, 312
Argininosuccinate,
  amphibian differentiation and, 61
  aspartate and, 150
Argyrotenia velutinana, DDT metabolism in, 440
Arion,
  ethereal sulfates and, 422
  phenols and, 418
Arionidae, sex hormone in, 271
Armadillidium, carotenoids in, 207
Aromatic acids,
  oxidation, induction of, 86
Aromatic compounds,
  detoxification of, 406–409, 427–432
  oxidation, copper and, 180
Aromia, salicin and, 405
Arsenic,
  cell growth and, 186
  selenism and, 185
Artemia, enzyme repression in, 99
Artemia salina,
  folic acid and, 200
  glutathione and, 195
Arthropods,
  acetylcholine in, 250, 251, 252
  chitin synthesis in, 163
  copper in, 180, 181
  enzyme repression in, 99
  ethereal sulfates in, 420, 445
  eye of, 206
  5-hydroxytryptamine in, 255
  inactive enzymes in, 104
  juvenile hormone in, 280
  neurosecretion in, 261–270
  nonneural hormones in, 271–282
  tapetum lucidum of, 164
  vitamin A in, 208
ω-Arylalkylcarboxylic acids, oxidation of, 405–406
Arylsulfatase(s), occurrence of, 422, 445
Ascaris,
  collagen of, 160
  rhodanese in, 434
Ascites cells, feedback inhibition in, 78
Ascorbate,
  adrenal, corticotropin and, 317
  biosynthesis of, 194
  electron transport and, 194
  folic acid and, 200
  insect hemolymph and, 140

need for, 172
Ascorbic acid oxidase,
  copper and, 181
  occurrence of, 194
Ashbya gossypii, riboflavin and, 189
Asparagine,
  detoxification and, 431
  importance of, 150
  requirement for, 150
Aspartate,
  arginine-vasopressin and, 310–311
  arginine-vasotocin and, 312
  larva and, 228
  lysine synthesis and, 155
  β-melanocyte-stimulating hormone and, 322
  oxytocin and, 309
  pantothenate and, 198
  requirement for, 159
  secretion of, 383
  synthesis, 75, 150
    abiogenic, 130, 131
  threonine synthesis from, 151
  transport of, 351–352
  ureidosuccinate formation from, 130
  use in biosyntheses, 150
Aspartate transcarbamylase,
  feedback inhibition of, 76, 78
  repression of, 96–97
Aspergillus,
  calcium and, 175
  methylation in, 426
Aspergillus niger,
  copper requirement of, 180
  gallium and, 186
  germination of, 9
  magnesium assay with, 176
  mineral requirements of, 137
  molybdenum and, 183
  phenoxyacetic acid and, 409
  potassium assay and, 174
  sodium requirement of, 174
  vanadium requirement of, 185
  zinc assay with, 178
Aspergillus oryzae, product inhibition in, 79
Astacus,
  chromatotropin of, 264
  intestinal absorption in, 355
Astaxanthin,
  crustacea and, 207
  formation of, 405
Asymmetry, prebiotic syntheses and, 131, 132
Atherosclerosis, essential fatty acids and, 194
Athiorhodaceae,
  enzyme induction in, 84, 86
  photosynthesis by, 143
Atropine, cholinergic system and, 249
Attagenus, amino acid requirement of, 160
Atya,
  fat absorption in, 355
  hepatopancreas, rhythmic secretion of, 385
Aureomycin,
  manganese and, 182
  occurrence of, 177
Avena sativa, rhodanese in, 434
Avidin, biotin and, 197
Avocado,
  succinic dehydrogenase, inhibition of, 80
8-Azaguanine, sporulation and, 18–19
Azaserine, purines and, 151
7-Azatryptophan, enzyme induction and, 83
Azotobacter,
  calcium and, 175
  iron requirement of, 179
  molybdenum and, 183
  permeases of, 85
  potassium and, 174
  ubiquinone of, 190
  vanadium requirement of, 185
Azotobacteriaceae, enzyme induction in, 84

B

Bacillaceae, enzyme induction in, 84, 86
Bacillariophyceae, vitamin B_{12} and, 203
Bacillus cereus,
  penicillinase, induction of, 85, 86
  pigment formation by, 138
  spores,
    germination of, 5, 7, 9, 11, 19
    penicillinase of, 17
  sporulation,

    amino acid pools and, 14
    potassium and, 174
    respiration and, 12–13
Bacillus megaterium,
  anthranilic acid and, 418
  germination,
    peptidases and, 9
    respiration and, 5–6
  vitamin B_{12} and, 202
Bacillus oligocarbophilus, free energy of, 145
Bacillus subtilis,
  amylase, ethionine and, 93
  enzyme derepression in, 96
  polysaccharide of, 162
  spermine and, 211
  spores, cytochrome in, 10
Bacteria, *see also* Microorganisms
  amino acid requirements of, 159
  calcium and, 137
  capsules, glutamate and, 150
  cellulose digestion and, 366, 385, 389
  comparative nutrition of, 122
  digestion by in fish, 376–377
  enzyme induction in, 83–85
  enzyme repression in, 95–98
  feedback inhibition in, 74–77
  germination,
    electron transport and, 10–11
    energy requirements of, 5–10
    initiation of, 5
  glycine requirement of, 149
  halophilic, chloride and, 177
  inactive enzymes in, 104–105
  iodine and, 177
  lysine synthesis by, 155
  magnesium requirement of, 176
  marsupial digestion and, 367
  nicotinamide synthesis in, 188
  Pasteur effect in, 81
  permease induction in, 85, 88
  pH optima of, 136
  potassium requirement of, 174
  product inhibition in, 79
  ruminant digestion and, 362–365
  sporulation,
    energy requirement for, 12–14
    initiation of, 11–12
  sterols in, 208
  thiamine and, 165
  wax digestion and, 388, 389
  zinc and, 178
Bacteriophage,
  deoxyribonuclease synthesis and, 83
  polyamines and, 211
Bacterium cadaveris,
  lysine decarboxylase, induction of, 86
Balanoglossida, juvenile hormone in, 281
Bankia, cellulase in, 387
Barbary dove, benzoic acid and, 429
Barbiturates, detoxification of, 405
Barium,
  calcium requirement and, 175
  ion antagonism and, 186
Barley,
  glycosides in, 416
  seeds, germination of, 31, 32
Barn owl, gizzard pH in, 370
Bartonella bacilliformis, porphyrin and, 190
Basidiomycete(s), enzyme induction in, 90
Batrachians,
  adrenals, mammalian corticotropin and, 317, 318
  antidiuretic activity in, 304, 305
  insulin action in, 332
  neurohypophyseal hormones of, 307–308
  posterior pituitary powder, activity of, 314
Bean(s),
  arbutin and, 406
  2,4-dichlorophenoxyacetic acid and, 406, 418
  nicotinuric acid in, 430
Bee,
  carbohydrate requirement of, 146, 147
  hemolymph sugars in, 141
  pheromone of, 288
  queen substance of, 288
  retinene in, 207
  sting, pheromone of, 288
Beef, *see also* Calf, Cattle, Cow
  corticotropin of, 320–321, 325–326
  glycine N-acylase of, 431
  insulin of, 333, 335–336
  liver, product inhibition in, 79
  α-melanocyte-stimulating hormone of, 319, 321

- β-melanocyte-stimulating hormone of, 319, 323, 324
- neurohypophyseal hormones, purification of, 304–307
- oxytocin of, 309
- posterior pituitary powder, activity of, 314
- vasopressin of, 311

Beeswax, digestion of, 372

Beet, thiocyanate in, 434

Beggiatoa, free energy of, 144

Begonia,
- sap, pH of, 136

Belostoma indica, Δ^2-hexenol acetate in, 287

Benzaldehyde, detoxification of, 404

Benzene, detoxification of, 433

Benzene hexachloride, dechlorination of, 438

Benzimidazolylcobamide, formation of, 201

Benzoic acid,
- derivatives, detoxification and, 404
- detoxification of, 415, 427–430, 442
- ornithine and, 427–428

1-O-β-Benzoyl-D-glucose, occurrence of, 162

Benzoylglucuronide, formation of, 428

Benzpyrene hydroxylase, induction of, 92

Benzyl alcohol, detoxification of, 404

Benzylbromide, detoxification of, 432

Beryllium,
- plants and, 137
- skeleton, formation and, 169
- zinc and, 186

Betaines, choline and, 204

Bicarbonate, osmotic balance and, 138, 139

Bile,
- amylase in, 371
- bird, pH of, 371
- DDT in, 438
- formation, ethionine and, 93

Bile acids,
- feedback inhibition and, 78
- glycine and, 431
- intestinal absorption and, 351
- proteinase activation by, 360
- sulfates of, 420

Biocytin, occurrence of, 196

Biopoietic demands, abiogenic fulfillment of, 128–135

Biopterin, activity of, 200

Biosphere,
- nutritional economy of, 126–128
- primitive, 129–130

Biotin,
- bacterial photosynthesis and, 143
- biosynthesis of, 196
- concentration of, 170
- cystine and, 153
- function of, 196
- lysine and, 155
- microorganisms and, 170
- requirement for, 197, 203

Biotin 1-sulfoxide, 197

Birds
- acetylation in, 424, 425
- adrenals, mammalian corticotropin and, 317
- amino acid requirement of, 162
- *p*-aminobenzoate and, 420
- arginine requirement of, 154
- arginine-vasotocin in, 316
- aromatic acids and, 429, 431
- bile, pH of, 371
- blood osmotic pressure of, 139
- choline and, 204
- corticotropin and, 318
- digestion in, 367–372
- ethereal sulfates in, 420, 421
- gastric pH in, 359
- glucuronides in, 414
- glycine requirement of, 149
- insulin action in, 332
- manganese requirement of, 182–183
- neurohypophyseal hormones,
  - activity in, 303, 305
  - purification of, 307
- nitro groups and, 413
- ornithuric acids and, 427–428
- posterior pituitary powder, activity of, 314
- protein requirement of, 159
- salicylic acid and, 428
- tocopherol and, 192
- vitamin A requirement of, 208

1,1-Bis(*p*-chlorophenyl)-2,2,2,-trichloroethanol, DDT metabolism and, 440, 441

Bis-1,3-β-ethyhexyl-5-methyl-5-amino-hexahydropyrimidine, spore germination and, 7
Bitter brush, deer and, 364
Blackbird, chitinase in, 361
Blacktongue, nicotinamide and, 188
Bladder,
  arginine-vasotocin and, 316
  hydroosmotic activity and, 303
Blaps,
  ethereal sulfates in, 421
  glucosides in, 415
Blastocladiales, lysine biosynthesis by, 124
Blastocladiella emersonii, differentiation of, 24
Blattella,
  copper and, 181
  nicotinamide and, 187
Blattella germanica,
  amino acid requirement of, 160
  calcium requirement of, 175
  choline and, 204
  manganese and, 182
  sodium and, 174
  zinc requirement of, 178
Blattoidea, disaccharases of, 383
Blood,
  clotting, calcium and, 175
  copper in, 180
  proteins, differentiation and, 56
  sugar, ruminants and, 367
Blowfly,
  chemoreceptors, repression of, 100
  extraintestinal digestion in, 388
  glucose absorption by, 355
Bombykol, chemical nature of, 286
Bombyx,
  brain hormone in, 267
  ecdysone isolation from, 234
  extracts, ecdysone in, 273
  glucosides in, 415
  glycogen in, 231
  methylation and, 426
  peritrophic membrane of, 382
  phosphorus in, 232
  trehalose in, 231
Bombyx mori,
  acetylation in, 424, 425
  aromatic acids and, 429
  chitinase in, 386
  DDT metabolism in, 440
  development, amino acids and, 227
  diapause hormone of, 269
  metamorphosis, respiration and, 224
  sex attractant of, 286
  zinc requirement of, 178
Bone, elements accumulated in, 187
Borate,
  surface water, origin of, 130
Boron, requirement for, 186
Borrelia,
  pyridine nucleotides and, 188
  thiamine and, 196
Botryllus, iodine fixation by, 284
Botrytis allii, fungicides and, 435
Bovidae, stomach of, 361
Brachiopoda, neurosecretion in, 257
Brain,
  cholinesterase, succinoxidase and, 59–60
  ergothionine in, 156
  neurosecretory cells of, 266–267
  product inhibition in, 78
  proteins, differentiation and, 56
Brain hormone,
  chemistry of, 267–268
  historical background, 266
  metamorphosis and, 222–223, 265
Branchial bodies, function of, 271
Brassicae, goitrogen in, 178
Briza media, glycosides in, 416
Brokeback, fluoro fatty acids and, 176
Bromide,
  amylase and, 124
  chloride requirement and, 177
  requirement for, 177
Bromoacetic acid, detoxification of, 433
Bromotyrosine, occurrence of, 160
Brush border, intestinal surface and, 349
Bryozoa, neurosecretion in, 257
Buccinum,
  acetylcholine effect on, 250
  5-hydroxytryptamine in, 255
Bufo, ethereal sulfates in, 421
Bufo bufo,
  acetylation in, 424
  glucuronides in, 414
Bufo marinus,
  dealkylation by, 411

glucuronides in, 414

Bufo valliceps, acetylation in, 424

Bugula flubellata, juvenile hormone in, 280

Busycon canaliculatum, 5-hydroxytryptamine in, 255

Butyrate,
  oxidation, induction of, 89
  ruminant and, 362

Butyribacterium rettgeri, lipoate and, 199

Butyryl coenzyme A dehydrogenase, copper and, 180

Byssus,
  fibers, iodine and, 284

C

Cabbage, thiocyanate in, 434

Cacodylic acid, methylation of, 426

Cadaverine, polyamine requirement and, 210–211

Cadmium, zinc and, 186

Caenorhabditis brigesae, folic acid and, 200
  nicotinamide and, 188

Caiman, aromatic acids and, 429

Calciferol, activity of, 209

Calcium,
  absorption, vitamin D and, 209
  hepatopancreas and, 382
  manganese requirement and, 182
  osmotic balance and, 138, 139
  plants and, 137
  potassium requirement and, 175
  ratio to magnesium, 176
  requirement, 125, 128, 175
    skeleton and, 168
  sea urchin eggs and, 40
  surface water, origin of, 130
  zinc deficiency and, 179

Calcium carbonate, skeletons and, 168

Calcium dipicolinate, spore germination and, 9

Calcium phosphate, skeletons and, 168

Calf, *see also* Beef, Cattle, Cow
  fetal, hippuric acid in, 445
  hippuric acid in, 427
  phenothiazine and, 409

Calliphora,
  brain hormone in, 268
  ecdysone assay and, 272, 273
  ecdysone formation by, 274
  gonadotropic hormone in, 279, 282
  metamorphosis, dehydrogenases and, 225–226
  phenoloxidase of, 236
  phosphorus in, 232

Calliphora erythrocephala,
  amino acids in, 229
  ecdysone action in, 275, 276
  metamorphosis, respiration and, 224
  peptides in, 229
  sclerotization in, 234

Calliphora vicina, amino acid requirement of, 161

Calvin cycle,
  enzymes, induction of, 84

Camel,
  glucuronides in, 414
  hippuric acid in, 427
  vasopressin of, 307

Camelidae, stomach of, 361

Cancer pagurus,
  chitinase in, 386
  emulsifier of, 383–384

Candida, enzyme induction in, 90

Capsules, polysaccharide synthesis and, 162

Carabus, extraintestinal digestion in, 388

Carausius,
  color change in, 270
  ethereal sulfates in, 421
  glucosides in, 415

Carbachol, pepsin secretion and, 373

Carbamyl phosphate synthetase, amphibian differentiation and, 60–63, 66–67, 107

O-Carbamyl-D-serine, formation of, 151

Carbohydrases, intestinal action of, 360

Carbohydrates,
  amphibian differentiation and, 53–54
  insect blood and, 141
  metabolism, metamorphosis and, 231
  requirement for, 146, 213
  sea urchin egg development and, 41
  seed germination and, 33, 34
  slime mold differentiation and, 22–24
  spore germination and, 8
  synthesis, potassium and, 174
  thiamine and, 196

- water metabolism and, 139
- Carbon,
  - amount in biosphere, 126
  - balance, maintenance of, 126–127, 147
- Carbonate,
  - surface water, origin of, 130
- α-Carbon atom,
  - labilization, vitamins and, 197–198
- Carbon dioxide,
  - acceptor, photosynthesis and, 142
  - enzyme induction by, 84
  - fixation, nonphotosynthetic, 143–148
  - formation and fixation, vitamins and, 195–197
  - Mars and, 134
  - marsupial digestion and, 367
  - primitive biosphere and, 130
  - reduction of, 144
  - rumen and, 362
  - sea urchin egg development and, 42
  - skeleton formation and, 169
  - succinic dehydrogenase inhibition by, 80
  - Venus and, 134
  - volcanic gas and, 130
- Carbonic anhydrase, zinc and, 178
- Carbon monoxide,
  - diapause and, 225
  - sea urchin egg and, 39–40
  - utilization of, 145
  - volcanic gas and, 130
- Carbostyrils, formation of, 412
- Carboxydismutase, induction of, 86, 88
- Carboxylic acids, detoxification of, 419
- Carboxypeptidase,
  - activation of, 105
  - insulin activity and, 336–337
  - β-melanocyte-stimulating hormone and, 322
  - zinc and, 178
- *Carcinides maenas*, juvenile hormone in, 280
- *Carcinus*, chromatotropins and, 264
- Cardiac gland zone, food fermentation and, 360
- Carmine, phagocytosis and, 378, 379
- Carnitine,
  - choline requirement and, 204
  - deficiency, zinc and, 178
  - function of, 205
- Carnivores,
  - gastric pH in, 359
  - intestinal pH in, 360
  - tapetum lucidum of, 164
- Carnosine, histidine and, 156
- Carotene,
  - formation,
    - copper and, 180
    - manganese and, 182
    - mevalonate and, 206
  - oxidation of, 405
- Carotenoids,
  - leucine and, 152
  - salt and, 138
  - solar energy and, 142
  - vitamin A and, 206
- Carrageenin, sulfate and, 420
- Carrot(s), hexachlorocyclohexane and, 438
- Casein,
  - hydrolyzed, growth promotion by, 211
- Castor bean,
  - germination of, 32, 34–36
  - succinic dehydrogenase, inhibition of, 80
- Cat,
  - 2-acetamidofluorene and, 407
  - acetylation in, 423, 424
  - amino acid transport in, 351
  - aniline and, 407
  - chitinase and, 361
  - chlorobenzene and, 407
  - glucuronides and, 414
  - glucuronyltransferase and, 420
  - hippuric acid in, 427
  - mercapturic acids in, 432
  - β-naphthylamine and, 407
  - nicotinic acid requirement of, 158, 187
  - rhodanese in, 434
  - uterus, oxytocic activity and, 302
  - vitamin A and, 207
- Catalase,
  - amphibian differentiation and, 58
  - induction of, 85, 86, 89, 90
  - inhibitor of, 51
  - iron and, 179
  - porphyrins and, 189
  - sea urchin egg development and, 50–51, 66

- spores and, 17, 18
- Catalysts,
  - evolution of, 213
  - polymerization and, 132
  - requirement,
    - acyl group transfer and, 198–199
    - basic concepts, 169–173
    - α-carbon atom and, 197–198
    - carbon dioxide and, 195–197
    - carotenoids and, 205–208
    - electron transfer and, 187–195
    - miscellaneous, 210–211
    - single-carbons and, 199–205
    - steroids and, 208–210
    - trace elements and, 173–187
- Catechol,
  - cleavage of, 409
  - detoxification of, 416
  - naphthalene and, 408
- Cathepsin,
  - amphibian differentiation and, 58
  - stomach and, 357–358
- Cations,
  - electrocardiograms and, 138–139
  - requirement for, 136
- Cattle, *see also* Beef, Calf, Cow
  - insecticides and, 444
  - phenothiazine and, 409
- *Cecidomya*, extraintestinal digestion in, 389
- Cecum, digestion and, 366, 372
- Cell membranes,
  - choline and, 204
  - essential fatty acids and, 193
- Cellulase,
  - crystalline style and, 384
  - induction of, 89, 90
  - occurrence of, 147, 391
- Cellulose,
  - digestion, 385–387
    - birds and, 372
    - rabbit and, 366
  - plant rigidity and, 167
  - seed germination and, 32
  - slime mold differentiation and, 24, 27
  - synthesis of, 162, 163
- Cell walls, lysine and, 155
- Cephalins,
  - serine and, 151
  - synthesis of, 163
- Cephalocordata, thyroid hormone in, 285
- Cephalopods,
  - acetylcholine in, 250
  - digestive secretions of, 382
  - endocrine glands in, 271
  - eye, 206
    - carotenoids in, 207
  - heart, adrenaline and, 254
  - neurosecretion in, 261
  - phagocytosis and, 381
  - salivary glands of, 382
  - venom, serotonin in, 158
- *Cephalopodes octopodes*, 5-hydroxytryptamine in, 255
- Cerebral gland, neurosecretion and, 261
- Cerebral hemisphere, intestinal absorption and, 354
- *Cerebratulus lacteus*, juvenile hormone and, 280
- Cerebroid ganglia, neurosecretion of, 259–260, 267
- Cerebrosides, synthesis of, 164
- Ceruloplasmin, copper in, 180
- *Cerura vinula*,
  - ecdysone action in, 275, 276
  - extracts, ecdysone in, 273
  - ommochromes in, 233
- Cervidae, stomach of, 361
- Cesium,
  - effects of, 186
  - potassium requirement and, 174
- Cestoda, gut of, 389
- *Cetraria islandica*,
  - hemicellulose, digestion of, 385
- *Chaetopterus variopedatus*, juvenile hormone in, 280
- *Chalcides ocellatus*, aromatic acids and, 430
- Cheilostomata, juvenile hormone in, 280
- Chemosynthetic autotrophs,
  - amino acid requirements of, 158
  - free energy of, 143–146
- Chick, *see also* Hen
  - alkaline, phosphatase, differentiation and, 57
  - *p*-aminobenzoate deficiency in, 201
  - arginine requirement of, 154
  - aromatic acids and, 429, 430
  - cardiac myosin, differentiation and, 57
  - depressor activity in, 303, 305

  embryo,
    arginase in, 51, 66
    enzyme induction in, 92
    ethereal sulfates in, 444–445
    feedback inhibition in, 78
  enzyme differentiation in, 2
  enzyme repression in, 100–101
  gastric secretion,
    pepsin in, 371
    stimulation of, 370–371
  gizzard contractions in, 367
  glycine synthesis by, 149, 432
  inactive enzyme in, 104
  lysine requirement of, 155
  oxytocic and antidiuretic activities in, 303, 305
  tocopherol and, 192
  xanthopterin and, 200
  zinc deficiency in, 179
Chicken, *see also* Fowl, Hen
  acetylation in, 424
  amino acid requirement of, 162
  p-aminosalicylic acid and, 427
  demethylation by, 411
  fibroblasts, amino acid requirement of, 162
  gizzard pH in, 370
  hippuric acid in, 427
  liver, product inhibition in, 79
  ornithine N-acylase of, 431
Chilomonas, vitamin B_6 and, 197
Chilopoda,
  endocrine glands in, 271
  neurosecretion in, 261, 262
Chimpanzee, phenylacetic acid and, 427
Chinook salmon,
  amino acid requirement of, 162
  protein requirement of, 149
Chironomus, enzyme repression in, 99
Chironomus tentans,
  chromosomes, development and, 237
  ecdysone action in, 275
Chitin,
  digestion of, 385–386
  formation,
    nitrogen balance and, 127
    requirements and, 162–163
  peritrophic membrane and, 382
  sclerotization and, 234
Chitinase,
  lizard and, 373
  mammals and, 361
  occurrence of, 147
Chitobiase,
  hepatopancreas and, 386
  secretion of, 361
Chlamydomonas,
  amino acid requirement of, 158
  hydrogenase of, 88
  sodium requirement of, 174
Chloramphenicol,
  algal hydrogenase and, 88
  deoxyribonuclease synthesis and, 83
  enzyme depression and, 96
  enzyme induction and, 82
  hydrolysis of, 436
  occurrence of, 177
  reduction of, 413
Chlorella,
  carboxydismutase, induction of, 88
  molybdenum and, 183
  potassium and, 174
Chloride,
  amylase and, 124
  function of, 171
  insect blood and, 140
  osmotic balance and, 138, 139
  plants and, 137
  requirement for, 177
  rumen and, 363
  surface water, origin of, 130
Chloridella empusa, skeleton of, 168
Chlorobacteriaceae,
  photosynthesis, hydrogen donor and, 142
Chlorobenzene,
  dechlorination of, 436
  detoxification of, 433
  oxidation of, 406, 407
p-Chlorobenzoic acid, DDT metabolism and, 440, 441
Chlorobium thiosulfatephilum, photosynthesis by, 142
Chlorobromomethane, dehalogenation of, 437
Chlorocarbon compounds, occurrence of, 177
Chlorocruorins,
  iron and, 179
  porphyrins and, 189

Chloroethanol, detoxification of, 417
Chlorogonium elongatum, hydroxyapatite in, 168
Chloromuconic acid, dechlorination of, 436–437
Chloromycetin, *see* Chloramphenicol
o-Chlorophenol, detoxification of, 417, 419
Chlorophenoxyacetic acid,
  dechlorination of, 436–437
  oxidation of, 408–409
p-Chlorophenylmercapturic acid, excretion of, 433
Chlorophyceae, vitamin B_{12} and, 203
Chlorophyll,
  iron and, 179
  magnesium requirement and, 175, 176
  porphyrins and, 189
  solar energy and, 142
Chlorophyta, enzyme induction in, 88
Chloropromazine, detoxification of, 409, 446
Chlortetracycline, occurrence of, 177
Choloepus hoffmanni, stomach of, 367
Cholesterol,
  bile acid formation from, 78
  brain hormone and, 267
  ecdysone formation from, 274
  hepatopancreas and, 382
  requirement for, 209
  synthesis, repression of, 101–102
Cholesterol esterase, sterol utilization and, 209
Choline,
  biosynthesis of, 203, 204
  function of, 203
  lipid synthesis and, 163
  metabolism, manganese and, 182
  requirement for, 204–205
  serine and, 151
Cholinesterase,
  amphibian differentiation and, 58–60
  cholinergic system and, 249
  distribution of, 249–250
Choline oxidase, methionine and, 204
Chondrodystrophy, manganese and, 183
Chondroitin, sulfate of, 420
Chromatophores, classification of, 263
Chromatotropins,
  chemical nature of, 263–264
  production of, 262
  purification of, 264–265
  theories regarding, 263
Chromium, effect of, 186
Chromosomes, ecdysone and, 237–239, 276–277
Chromatophoric hormone, subneural gland and, 283
Chrysanthemumic acid, formation of, 434–435
Chrysophyceae, vitamin B_{12} and, 203
Chrysophyta, enzyme induction in, 88
Chymosin, pH optimum of, 358
Chymotrypsin,
  activation of, 105
  chromatotropins and, 264
  corticotropin and, 325
  α-melanocyte-stimulating hormone and, 321
  β-melanocyte-stimulating hormone and, 322
Chytriales, lysine biosynthesis by, 124
Cilia, digestive movements and, 381
Ciliates,
  amino acid requirement of, 160
  skeletons of, 168
Cimbex americana,
  diapause in, 269
  ecdysone action in, 275
Ciona, iodine fixation by, 284
Citrate,
  germinating seeds and, 37
  insect blood and, 141
  permease for, 85
Citric acid cycle,
  enzymes, induction of, 19, 86
  intestinal absorption and, 353
  permeases and, 85
  spores and, 15
Citrovorum factor, *see* Folic acid
Citrulline,
  arginine and, 154
  aspartate and, 150
Citrus trees, zinc deficiency in, 178
Cladocerans, enzyme repression in, 99
Clemmys capsica, digestive secretion of, 373
Cliona celata, juvenile hormone and, 280
Clonothrix, free energy of, 145

Clostridium, molybdenum and, 183
Clostridium aceticum, hydrogen utilization by, 144
Clostridium tetani,
  adenine and, 165
  oleate and, 193
Clostridium tetanomorphum, adenylcobamide of, 201
Clothes moths, silk digestion by, 388
Cnidaria, juvenile hormone in, 280
Cobalamin, *see* Vitamin B_{12}
Cobalt,
  copper deficiency and, 181
  function of, 171
  requirement for, 184–185
  spore germination and, 9
Cobra,
  venom, zinc and, 178
Cocarboxylase, *see also* Thiamine pyrophosphate
  nonenzymic catalysis by, 171
  space germination and, 7
Coccinella, glucosides in, 415
Coccolithophoridase, skeletons of, 167
Cockroach,
  acethion and, 444
  alanine requirement of, 150
  aldrin and, 411
  chloride requirement of, 177
  cystine and, 153
  gonadotropic hormone in, 279
  hormonal effects in, 269
  malaoxon and, 443
  mercapturic acids in, 433
  periplanetin of, 162
  phenothiazine and, 406–407
  phenylalanine and, 156
  serine requirement of, 151
  threonine and, 151
Cocoon,
  spinning, free amino acids and, 227–228
Cod, rhodanese in, 434
Codonosiga botrytis, food uptake by, 379
Coelenterates,
  acetylcholine and, 248, 249, 251
  5-hydroxytryptamine in, 255
  neurosecretion in, 257
  phagocytosis in, 380
Coenzyme A, 164
  amino acid conjugation and, 430
  lipoate and, 199
  pantothenate and, 198
  requirement for, 196, 199
  thiamine and, 195
Coffee,
  beans, manganese in, 182
Coilin, gizzard and, 367
Colchicine,
  toxicity, temperature and, 441
Coleoptera,
  amino acid requirement of, 160
  glucosides in, 415
  hemolymph, ions in, 140
  juvenile hormone in, 278, 280
  metamorphosis, respiration and, 224
Collagen,
  ascorbate and, 194
  digestion of, 388
  glycine requirement and, 149
  hydroxylysine and, 154
  hydroxyproline and, 153
Collagenase(s), calcium and, 175
Collateral glands, gonadotropic hormone and, 279
Colon,
  amino acid transport in, 352
  digestion and, 366
Colorado beetle, diapause in, 269
Colpoda, vitamin B_6 and, 197
Comparative nutrition,
  biochemical function and, 122
  contemporary status of, 120–122
  genetic capabilities and, 123–125
  quantitative aspects, 125–126
  reviews of, 122
Conchiolin, mollusk shells and, 168
Conchostracans, enzyme repression in, 99
Conjugation, detoxification and, 404, 413–433, 447–448
Convulsive action, insulin and, 331, 338
Copepoda, gut of, 389
Copper,
  molybdenum deficiency and, 184
  requirement for, 128, 180–181
  toxicity of, 180–181
Coprogen, requirement for, 190
Corals,
  dibromotyrosine in, 177
  diiodotyrosine in, 177

  iodine fixation by, 284
  skeletons of, 168
Corcyra,
  copper and, 181
  methylation and, 426
Corcyra cephalonica,
  aromatic acids and, 429
  enzyme induction in, 91
  iodoamino acids and, 178
  nicotinamide and, 187–188
  vitamin B_6 and, 198
  zinc requirement of, 178
Corethra, melanophores of, 270
Corn, *see also* Maize
  glycosides in, 416
  nicotinic acid in, 158
Corpora allata,
  gonadotropic hormone of, 279, 282
  hormones of, 223, 265, 266, 272
  insectoverdin and, 234
  juvenile hormone and, 277
  neurosecretion and, 261, 267
Corpus cardiacum,
  myotropic activity and, 269
  neurosecretions and, 261, 266, 267
Corticosteroids, corticotropin and, 317–318
Corticotropin,
  acetylated, melanocyte stimulation by, 328
  adrenal ascorbate and, 317
  chemical synthesis of, 325, 327
  derivation of, 301
  α-melanocyte-stimulating hormone and, 325, 327
  purification of, 320–321
  structure, 325
    α-melanocyte-stimulating hormone and, 327
Cortisol, enzyme induction and, 92–93
Corynebacteriaceae, enzyme induction in, 84
Corynebacterium, permeases of, 85
Corynebacterium diphtheriae,
  iron requirement of, 179
  oleate and, 193
Cossus, wood utilization by, 387
Cotinine, formation of, 412
Cotton,
  seed, germination of, 32
Coumarins, tyrosine and, 156
Cow(s), *see also* Beef, Calf, Cattle
  acetylation in, 423
  glucuronides in, 414
  hippuric acid in, 427
  juvenile hormone in, 281
  rhodanese in, 434
Crab(s), N-methylpyridinium hydroxide in, 426
Crago septemspinosus, juvenile hormone in, 280
Crangon crangon, chromatotropin of, 264
Creatine,
  enzyme repression by, 100–101
  glycine and, 150
Crenothrix, free energy of, 145
Crithidia fasciculata,
  amino acid requirement of, 160
  biopterin and, 200
  methyl donors and, 204
  porphyrin and, 190
  purine requirement of, 166
  threonine requirement of, 151, 152
Crocodile, antidiuretic activity in, 303
Crop, digestion and, 367, 368
Crow, ethereal sulfates in, 421
Crude fiber, digestion of, 367, 372
Crustacea,
  acetylcholine in, 249, 250
  carotenoids of, 206, 207
  chromatophores of, 263
  color change hormones of, 270
  comparative nutrition of, 122
  emulsifiers in, 383–384
  endocrine glands in, 271–272
  enzyme repression in, 99
  heart, adrenaline and, 254
  hepatopancreas in, 381
  5-hydroxytryptamine in, 255, 256
  intestinal absorption in, 355
  molting of, 168–169
  neurosecretion in, 262–265
  phagocytosis and, 381
  skeletons of, 168
  tocopherol and, 192
  uric acid in, 140
Cryptocercus, ecdysone action in, 275, 277
Cryptophyceae, vitamin B_{12} and, 203
Cryptoxanthin, vitamin A and, 206

Crystalline style,
  composition of, 384
  enzymes in, 384
Ctenolepisma lineata, cellulase of, 386
Ctenophora, neurosecretion in, 257
Ctenostomata, juvenile hormone in, 280
Culex fatigans,
  iron requirement of, 179
  sodium and, 174
Culture methods, 121
Cuproporphyrin, occurrence of, 180
Cura foremani, juvenile hormone and, 280
Curare, cholinergic system and, 249
Cuticle, formation of, 229
Cyanide,
  aminoacetonitrile formation and, 132
  detoxification of, 433–434
  diapause and, 225
  sea urchin eggs and, 39
  spore germination and, 10–11
Cyanophyta, enzyme induction in, 88
Cycloserine, purines and, 151
Cyclostome,
  larva, thyroid hormone in, 284, 285
Cyprinidae, digestive tract of, 376
Cyprinodontidae, digestive tract of, 376
Cystathionine, serine and, 151
Cysteine,
  arginine-vasopressin and, 310–311
  arginine-vasotocin and, 312
  biotin and, 196
  dehalogenation and, 436
  detoxification and, 432–433
  insulin and, 333–334
  methionine requirement and, 153
  oxytocin and, 309
  pantothenate and, 198
  requirement for, 152, 159–163
  selenium and, 185
  serine and, 151
  synthesis, repression of, 96
  utilization of, 153
Cysteinylglycine, DDT dehydrochlorinase and, 439
Cytidine, assay of, 165
Cytidine triphosphate, aspartate transcarbamylase and, 76
Cytidylic acid,
  aspartate transcarbamylase and, 76
  requirement for, 166
Cytochrome(s),
  chemoautotrophs and, 143
  induction of, 19
  iron and, 171, 179
  porphyrins and, 189
  repression of, 99, 100
  sea urchin egg development and, 38–40
  spores and, 10
Cytochrome(s) a,
  metamorphosis and, 224
  induction of, 86
Cytochrome b,
  metamorphosis and, 224
  vitamin K and, 191
Cytochrome b_5, metamorphosis and, 224, 225
Cytochrome c,
  essential fatty acids and, 193
  induction of, 90
  metamorphosis and, 224
Cytochrome c_1, quinones and, 190
Cytochrome oxidase,
  amphibian differentiation and, 58, 59, 65
  essential fatty acids and, 193
  induction of, 90
  iron and, 179, 180
  metamorphosis and, 224–225
  sea urchin egg development and, 39–40, 50–51, 65
  seed germination and, 31
  spores and, 10
Cytochrome peroxidase, induction of, 89
Cytosine,
  assay of, 165
  requirement for, 165–166

D

Dandelion, glycosides in, 416, 418
Daphnia,
  enzyme repression in, 99
  pantothenate and, 199
  tocopherol and, 192
Dark reaction, photosynthesis and, 142
DCDNS, nitrate reductase formation and, 91
DDT,
  dechlorination of, 437, 438

phenolic metabolite of, 406
toxicity, temperature and, 442
DDT dehydrochlorinase, cysteine and, 433, 439
Deacylase(s), occurrence of, 423–425
Decane, oxidation of, 146
Decapoda,
androgenic gland in, 271
emulsifiers in, 383–384
food absorption in, 355
Decarboxylases, induction of, 83
Deer, digestion in, 364
Dehalogenations, detoxification and, 436–441
7-Dehydrocholesterol,
sterol requirements and, 209
vitamin D and, 209
Dehydrogenase(s),
differentiation and, 28
induction of, 83
iron and, 179
metamorphosis and, 225–226
Deilephila euphorbiae, trehalose in, 231
Demyelinating diseases, copper and, 181
Dendrodoa, iodine fixation by, 284
Deoxyribonuclease,
activation of, 103
inactive form of, 104
manganese and, 182
synthesis, phage and, 83
Deoxyribonucleic acid,
amphibian differentiation and, 58
growth antagonism by, 166
metamorphosis and, 232
polysaccharide synthesis and, 162
spermine and, 211
sporulation and, 18
Deoxyribosides, assay of, 165
Depressor activity, birds and, 303, 305
Dermestes vulpinus, sterol requirement of, 209
Deroceras agreste, juvenile hormone in, 280
Desmospongiae, juvenile hormone and, 280
Desthiobiotin, activity of, 196
Desulfovibrio,
hydrogen utilization by, 144
salt adaptation of, 137
Detoxification,
acetylation and, 423–425
cysteine and, 432–433
definition of, 403
dehalogenation and, 436–441
developmental aspects of, 444–447
ethereal sulfates and, 420–423
hydrolysis and, 434–436
mechanisms of, 404–441
methylation and, 425–427
oxidations and, 404–412
peptide conjugation and, 427–432
reaction rates and species differences, 441–444
reduction and, 413
syntheses or conjugations and, 413–433
terrestrial habitat and, 447–448
thiocyanate formation and, 433–434
Dextran,
degradation, induction of, 89
synthesis of, 162
Dextrin, seed germination and, 33
α,ϵ-Diaminopimelate,
cell walls and, 155
lysine biosynthesis and, 124
spore germination and, 9–10
2,6-Diaminopurine, sporulation and, 18–19
2,4-Diamino-2,4,6-trideoxyhexose, occurrence of, 162
Diantline, inhibition of, 261
Diapause,
hormonal control of, 266
occurrence of, 222
Diapause hormone, formation of, 269
Diaphorase, spores and, 11
α,δ-Dibenzoylornithine, formation of, 428
Dibromoindigo,
formation of, 422
occurrence of, 177
Dibromotyrosine,
occurrence of, 177
tyrosine and, 156
Dichlorbenzhydrol, DDT metabolism and, 440
Dichlorobenzenethiols, formation of, 433
Dichlorobenzophenone, DDT metabolism and, 440, 441

2,3-Dichloroisobutyrate, pantothenate and, 199
2,4-Dichlorophenoxyacetic acid, detoxification of, 406, 418
Dicotyledons, enzyme induction in, 90
Dictyosteliaceae,
  fruiting, glucose and, 163
Dictyostelium, enzyme activation in, 104
Dictyostelium discoideum,
  differentiation,
    histidine and, 26–27
    respiration and, 21, 27
  endogenous reserves of, 64
Dicumarol, vitamins K and, 191
3,6-Dideoxyaldohexoses, occurrence of, 162
Dieldrin, formation of, 411
Differentiation,
  enzyme controls and, 107
  initiation and control of, 63–64
Digestion,
  absorption process and, 349–356
  course in fish, 376–377
  extraintestinal, 388–389
  general aspects of, 347–356
  intracellular, 378–381
  special foods and, 385–388
  special groups of vertebrates and, 356–377
  symbiosis and, 389–390
Digestive tract, 5-hydroxytryptamine in, 255
Dihydrodihydroxydiols, formation of, 407, 411
S-(1,2-Dihydro-2-hydroxy-1-naphthyl) glutathione, formation of, 433
Dihydro-orotase, feedback inhibition of, 78
Dihydroxyphenol, myotropic activity and, 269
Dihydroxyphenylalanine, formation of, 235
3,4-Dihydroxyphenyl glucoside, formation of, 406
Dihydroxyphenylpyruvate, *o*-diphenoloxidase and, 236
Diiodotyrosine,
  occurrence of, 177–178
  tunicates and, 284, 285
  tyrosine and, 156
Diisopropylfluorophosphonate, detoxification of, 436
2,3-Dimethoxy-5-methylbenzoquinones, electron transport and, 190–191
1,4-Dimethoxyqnaphthalene, fungicidal activity of, 435
5,6-Dimethylbenzimidazole, vitamin B_{12} and, 201
Dimethylbenzimidazolylcobamide, isolation of, 201
Dimethylethanolamine, 204
Dimethyl-*p*-phenylenediamine, sea urchin eggs and, 40
Dimethylpropylarsine, formation of, 426
Dimethylselenide, formation of, 427
Dinitrochlorobenzene, detoxification of, 432
Dinitrocresol, sea urchin eggs and, 41
Dinitrofluorobenzene, insulin structure and, 333
Dinitrophenol,
  amino acid absorption and, 351, 352
  germinating seeds and, 37
  sea urchin eggs and, 41
  slime mold respiration and, 27
Dinophyceae, vitamin B_{12} and, 203
Dionaea, prey capture by, 390
Dipeptidase, amphibian differentiation and, 58
Dipeptides, intestinal absorption of, 351
Diphenacetylornithine, formation of, 427
o-Diphenol(s), tanning and, 234
o-Diphenoloxidase, specificity of, 236
1,3-Diphosphoglyceric acid, photosynthesis and, 142
Diphosphopyridine nucleotidase, repression-like effects on, 104
Diphosphopyridine nucleotide, 164
  electron transport and, 187
  glycine formation and, 149
  lipoate and, 199
  nutritional requirement for, 196
  photosynthesis and, 142
  reduced, spores and, 18
  sea urchin eggs and, 48, 49
  spore germination and, 11
  synthesis, repression of, 96
  vitamin A and, 207
  vitamin K and, 191

Diphosphopyridine nucleotide oxidase,
  spore germination and, 11, 16
  sporulation and, 18
Dipicolinate,
  enzyme stability and, 17
  respiration and, 10–11, 18
  spore germination and, 9–10
  sporulation and, 14
Diplococcus pneumoniae, choline and, 204
Diplopoda,
  endocrine glands in, 271
  neurosecretion in, 261, 262
Dipnoi, digestive tract of, 376
Diptera,
  differentiation in, 222
  ecdysone action in, 275
  extracts, ecdysone in, 273
  glucosides in, 415
  hemolymph, ions in, 140
  juvenile hormone in, 280
  puparium formation, amino acids and, 229
Disaccharases,
  insect, 383
  loss of, 389
Disaccharides,
  hydrolysis, intestine and, 353
6,8-Dithiooctanoic acid, *see* Lipoic acid
Dixippus, color change in, 270
Dog(s),
  2-acetamidofluorene and, 407
  acetylation in, 423, 424
  amino acid requirement of, 163
  amino acid transport in, 351
  p-aminosalicylic acid and, 427
  aniline and, 407
  antidiuretic activity and, 303, 305
  aromatic acids and, 430, 442
  DDT metabolism in, 438
  enzyme induction in, 92
  ethereal sulfates in, 420
  heptachlor and, 411
  hexobarbital and, 405
  hippuric acid in, 427
  mercapturic acids in, 432
  β-naphthylamine and, 407
  rhodanese in, 434
  saliva, amylase in, 357
  vasopressin of, 307
Dogfish,
  course of digestion in, 377
  gastric juice, secretion of, 374
  gastric pH of, 359
  pituitary powder activity of, 314
Dolichoglossus kowalevskii, iodide fixation by, 282–283
Dolium, digestive secretion of, 383
Dopamine,
  o-diphenoloxidase and, 236
  occurrence of, 248
Double gradient, sea urchin egg development and, 45–47, 66
Dove(s), benzoic acid and, 428
Drosera, proteolysis by, 390
Drosophila,
  calcium and, 175
  carnitine and, 204
  copper and, 181
  glucosides in, 415
  metamorphosis, cystine and, 152
  nicotinamide and, 187
  phenoloxidase of, 236
  sugar absorption in, 355
Drosophila melanogaster,
  amino acid requirement of, 161
  DDT metabolism in, 440, 441
  ecdysone action in, 275
  purine requirement of, 167
  sodium requirement of, 174
Duck(s)
  benzoic acid and, 428, 429
  gizzard pH in, 370
  ornithine N-acylase of, 431
Ducklings,
  enzyme repression in, 100–101
  tocopherol and, 192
Dunaliella salina, pigment of, 138
Dytiscus,
  enzyme secretion in, 385
  extraintestinal digestion in, 388–389
  glucosides in, 415

E

Earthworms, cellulase in, 147
Ecdysone,
  bioassay of, 272–277
  brain hormone and, 266
  chemical properties of, 273–274
  chromosomes and, 237–239

  extraction and purification of, 273
  gland producing, 261–262
  history of, 272
  isolation of, 234
  metabolism of, 274
  metamorphosis and, 222–223, 265–266
  physiological action of, 274–277
  pigment synthesis and, 233
  ribonucleic acid and, 232
  sclerotization and, 234–237
Echinarachnius parma, skeleton formation in, 168
Echinochrome, iron and, 179
Echinoderms,
  acetylcholine in, 249–250
  hepatopancreas and, 381
  juvenile hormone in, 281
  phagocytosis in, 381
  skeletons of, 168
Echinoidea, juvenile hormone and, 281
Echinone, vitamin A and, 206
Ectoderm, mitochondria and, 45–47
Ecotprocta, juvenile hormone in, 280
Eel, vitamin A of, 206
Egg(s),
  drug oxidation and, 446–447
  ethereal sulfates and, 444
  homogenates, respiration of, 57
  ornithuric acid in, 445–446
  production, manganese and, 182–183
  rhodanese in, 444
  ripening, gonadotropic hormone and, 279
  sulfacetimide and, 447
  uric acid synthesis in, 431
  white, biotin and, 197
  yolk, gastric lipase and, 359
Elasmobranchs,
  osmotic balance in, 138, 139, 212
  pancreas of, 374
Electric discharge, amino acid formation and, 130, 131
Electron transport,
  seed germination and, 31
  sea urchin egg development and, 38–41
  spore germination and, 10–11
  vitamins and, 187–195
Eledone moschata, acetylcholine effect on, 250
Elephant,
  glucuronides in, 414
  hippuric acid in, 427
Embden-Meyerhof pathway, spores and, 15
Emulsifiers, fat absorption and, 351
Emulsin, detoxification and, 419
Emys europaeus, aromatic acids and, 429
Emys orbicularis, digestive secretion of, 373
Encephalomalacia, tocopherol and, 192
Encephalopathy, selenium and, 185
Endogenous reserves,
  amphibian differentiation and, 53–57
  germination and, 8–10
  sea urchin egg development and, 41–45
  seed germination and, 31–36
  slime mold sporulation and, 22–27, 63
  sporulation and, 13–14
  utilization, differentiation and, 63–64
Endoproteinase, gastric, 357–358
Endostyle, function of, 283–285
Endosymbionts, occurrence of, 389–390
Endotoxins, monosaccharides in, 162
Energy,
  differentiation and, 20–27, 52–57
  intestinal absorption and, 350–351
  sea urchin egg development and, 38–45
  seed germination and, 30–36
  specialization and, 4
  spore germination and, 5–10
  supply, control of, 79–81
Enolase,
  fluoride toxicity and, 177
  manganese and, 182
Entamoeba histolytica, sterol requirement of, 209
Enterobacteriaceae, enzyme induction in. 86
Enterokinase, cephalopod, 382
Enteropneusta,
  endocrinology of, 282–283
  juvenile hormone in, 281
Entomostraca, neurosecretion in, 262
Enzootic ataxia, copper and, 181
Enzyme (s),
  activation of, 103–105

- advantages of, 106
- altered,
  - amphibian differentiation and, 57–63
  - mechanisms of, 64–67
  - sea urchin egg development and, 47–52
  - seed germination and, 36–38
  - spores and, 14–15, 27–30
- amount, variations in, 81–82
- derepression of, 96
- digestive,
  - secretion of, 348–349
  - vertebrate and invertebrate, 382–384
- formation, control of, 81–103
- inactivation,
  - differentiation and, 28
  - seed germination and, 37, 64
- induction,
  - advantages of, 106
  - bacteria and, 83–85
  - general concepts of, 82–83
  - other organisms and, 85–88
  - permeases and, 80–88
  - spores and, 17–19
- preformed, spores and, 15–17
- repression,
  - bacteria, 95–98
  - other organisms, 98–103
- secretion of, 385

Enzyme activity,
- control,
  - energy-supplying pathways and, 79–81
  - feedback inhibition and, 74–78
  - product inhibition and, 78–79

Eobionts, catalysis in, 170–171

Epeira, ethereal sulfates in, 421

Ephemeroptera, corpora allata of, 267

Ephestia,
- essential fatty acids and, 193
- peptides in, 229
- tocopherol and, 192

Ephestia kühniella,
- development, amino acids in, 228
- ecdysone action in, 275, 276

Epicuticle, composition of, 234

Epilachna varivestis, DDT metabolism in, 440

Epinephrine, *see also* Adrenaline
- gastric secretion and, 370–371
- tyrosine and, 156

Epistellary body, function of, 271

Epoxidation, detoxification and, 411

Equilenin, excretion of, 300

Equilin, excretion of, 300

Eremothecium ashbyii, riboflavin and, 189

Ergosterol,
- sterol requirement and, 208, 209
- vitamin D and, 209

Ergot,
- alkaloids, tryptophan and, 157

Ergothionine,
- occurrence of, 156
- synthesis of, 156

Erinaceus europaeus, chitinase in, 361

Eriocheir, chitin digestion by, 386

Erythrocruorins, iron and, 179

Erythrocuprein, copper in, 180

Erythrocytes,
- copper in, 180
- ergothionine in, 156
- zinc in, 178

Erythrophores, chromatotropins and, 263, 264

Erythrose-4-phosphate, phosphoglucoisomerase and, 81

Escherichia coli,
- adenylcobamide and, 201
- aspartate transcarbamylase of, 76
- chloride and, 177
- deoxyribonuclease, activation of, 103
- enzyme induction in, 84
- enzyme repression in, 95, 96
- feedback inhibition in, 74–75
- folic acid and, 201
- β-galactosidase, induction of, 82–83, 85, 86
- infected, deoxyribonuclease in, 83
- iron requirement of, 179
- juvenile hormone in, 281
- lipoate and, 199
- mineral requirements of, 137
- Pasteur effect in, 81
- penicillin and, 436
- permease, induction of, 85
- product inhibition in, 78, 79
- protoplasts, spermine and, 211

  sodium requirement of, 174
  spermidine in, 211
  starved,
    amino acid pool of, 14, 23
    endogenous reserves of, 64
    respiration of, 28
  threonine deaminase of, 76
  thymine assay and, 165
  tryptophan synthesis in, 157
  vitamin B_{12} and, 203
Escrine, cholinergic system and, 249, 250
Esophageal groove, 361
  function of, 362
Esophagus, pepsin secretion by, 373
Esox lucius, sugar absorption by, 354
Esterases, detoxification and, 434–435, 443
Ethane, oxidation of, 146
Ethanol, *see also* Alcohol(s)
  acetate utilization and, 88–89
Ethanolamine,
  choline and, 203–204
  glycine requirement and, 149
  lipid synthesis and, 163
  serine and, 151
Ethereal sulfates,
  detoxification and, 414, 420–423, 448
  development and, 444–445
Ethionine,
  enzyme induction and, 83, 93
  glutaminase and, 103
  glycine transamidinase and, 100
  incorporation of, 93
Ethylenechlorohydrin, detoxification of, 419
Eubacteria, lysine biosynthesis by, 123–124
Euglena,
  enzyme induction in, 88
  ethanol utilization by, 88–89
  lysine biosynthesis by, 124
  vitamin B_{12} and, 203
Euglenineae, vitamin B_{12} and, 203
Euglypha, skeleton of, 167
Eurycotis, Δ^2-hexenol acetate in, 287
Evolution,
  neurohypophyseal hormones and, 313–316
  thyroid hormone and, 284, 289
Excitability,
  calcium and, 175
  potassium and, 175
Extraterrestrial bodies, nutrition on, 133–135
Exudative diathesis, selenium and, 185
Eye(s),
  riboflavin in, 189
  tryptophan requirement and, 157
  zinc in, 178
Eyestalks, juvenile hormone in, 280

F

Farnesol,
  gonadotropic hormone and, 282
  juvenile hormone and, 278, 281
Farnesyl diethylamine, juvenile hormone and, 281
Farnesyl methyl ether, juvenile hormone and, 281
Farnesyl phosphates, juvenile hormone and, 281
Farnesyl pyrophosphate, leucine and, 152
Fat,
  amphibian differentiation and, 53–55
  DDT metabolites in, 438–439
  gastric absorption of, 377
  hepatopancreas and, 382
  intestinal absorption of, 351, 355, 356
  metabolism, gonadotropic hormone and, 282
  seed germination and, 32–34
  utilization, energy and, 147
  water metabolism and, 139
Fat body, metamorphosis and, 222, 225–226
Fatty acids,
  cecum and, 366
  emulsifiers and, 383
  essential,
    electron transport and, 192–193
    requirement for, 193–194
  formation,
    hydrocarbons and, 146
    manganese and, 182
  hepatopancreas and, 382
  intestinal absorption of, 351
  marsupial digestion and, 367

  oxidation, induction of, 89
  photosynthesis and, 143
  polyunsaturated, tocopherols and, 192
  ruminant and, 362, 363
Feathers,
  arginine requirement and, 149
  glycine requirement and, 149
  lysine and, 155
  pigment, copper in, 180
Feedback,
  benefits of, 105
  differentiation and, 66
  isotopic competition and, 75
Feeding, molting and, 276
Ferret(s), chlorobenzene and, 407
Ferricyanide, chromatotropins and, 264
Ferritin,
  induction of, 94–95
  iron and, 179
Ferrous ions,
  oxidation, free energy and, 144, 145
Fibrin,
  digestion, stirring and, 356
Fibroblasts, amino acid requirement of, 162
Fish
  acetylation in, 424
  acetylsulfanilamide and, 424
  amino acid requirement of, 162
  appendices pyloricae in, 375–376
  arginine-vasotocin in, 315
  choline and, 204
  comparative nutrition of, 122
  corticotropin and, 318
  demethylation in, 411
  digestion in, 374–377
  ethereal sulfates and, 420, 448
  freshwater, vitamin A of, 206
  glucuronides and, 414, 448
  liver oil, vitamin D in, 209
  marine, vitamin A, of, 206
  neurohypophyseal hormones,
    activity in, 304, 313
    purification of, 308
  pancreas of, 374–375
  posterior pituitary powder, activity of, 314
  protein requirement of, 149, 159
  rhodanese in, 434
  stomach of, 376
  tapetum lucidum of, 164
  tocopherol and, 192
Flagellates,
  marine, requirements of, 203
Flatworms,
  neurosecretion in, 257–258
  rhabdocoel, hemoglobin in, 100
Flavin,
  synthesis, feedback and, 77
Flavin adenine dinucleotide, 164
  electron transport and, 189
  molybdenum and, 183
  spores and, 11
Flavin mononucleotide, electron transport and, 189
Flavobacterium, enzyme induction in, 86
Flavoprotein,
  dealkylation and, 411
  diapause and, 225
  iron and, 179
  nitroreductase and, 413
  seed germination and, 31
  spores and, 11
Flax,
  root, thiamine and, 164
  seed, germination of, 32
Fluid medium, maintenance of, 135–141
Fluke(s), rhodanese in, 434
Fluoride,
  accumulation of, 187
  requirement for, 167, 169, 171, 176
  toxicity of, 176–177
  surface water, origin of, 130
Fluoroacetic acid, formation of, 405
Fluoroapatite, dental caries and, 169, 171, 176
Fluoro fatty acids,
  detoxification of, 405
  occurrence of, 176
p-Fluorophenylalanine, enzyme induction and, 83, 91
Fly, *see also* Housefly
  acethion and, 444
  DDT and, 406
  DDT dehydrochlorinase of, 433
  heptachlor and, 411
  hexachlorocyclohexane and, 433, 437
  malathion and, 443
  mercapturic acids in, 432
  naphthalenes and, 407–408

pyrethrins and, 435
Folic acid,
analogs, uses of, 200
biosynthesis of, 200
choline and, 204
functional form of, 199
glutamate and, 150
microorganisms and, 170
requirement, modification of, 200
thymine and, 166
Folinic acid, ascorbate and, 194
Foraminifera,
prey capture by, 378
skeletons of, 167
Forebrain, cholinesterase in, 59, 60
Formaldehyde,
amino acetonitrile formation and, 132
amino acid formation and, 130
bacterial morphology and, 145
serine requirement and, 151
Formate,
abiogenic formation of, 131
bacterial morphology and, 145
histidine degradation and, 156
serine and, 151
Formylkynurenine, formation of, 158
Formylpteroic acid, 200
5-Formyltetrahydrofolic acid, *see* Folic acid
Fowl,
enzyme induction in, 92
posterior pituitary powder, activity of, 314
Fox, acetylation in, 423
Free energy, nutritional sources of, 141–148
Frog,
acetylsulfanilamide and, 424
amylases in, 374
arginine-vasotocin in, 316
aromatic acids and, 429, 430
aromatic oxidations in, 406
colchicine and, 441
demethylation in, 411
detoxification in, 404
glucuronides in, 414, 445
heartbeat, cations and, 139
hydroosmotic activity in, 303, 305
melanocytes,
beef β-melanocyte-stimulating hormone and, 324, 327
hog corticotropin and, 316
hog β-melanocyte-stimulating hormone and, 327
methylation in, 425
natriferic activity in, 304, 305
pepsin secretion in, 373
posterior pituitary powder, activity of, 314
rhodanese in, 434
tissues, acetylcholine analogs and, 253
Fructokinase,
potassium and, 174
product inhibition of, 79
Fructose,
chemoreceptors, repression of, 100
glycoside formation and, 418
insect blood and, 141
intestinal absorption of, 350, 351, 354, 355, 373
Fructose diphosphatase, activation of, 105
Fructose-1,6-diphosphate,
oxidation, sea urchin eggs and, 49
Fructose-6-phosphate, tryptophan synthesis and, 157
Fumarase, induction of, 90
Fumarate, insect blood and, 141
Fundulus,
melanocorticotropic hormones and, 318
osmoregulation in, 315–316
pilocarpine and, 374
spasmodic activity in, 304
Fungi, *see also* Microorganisms, Molds
aromatic oxidation in, 409
calcium and, 137
carnivorous, 390
comparative nutrition of, 122
enzyme induction in, 89–90
enzyme repression in, 98–99
ergothionine and, 156
inactive enzymes in, 104
iron and, 179
lysine biosynthesis by, 124
permease induction in, 90
phenolase of, 406
pH optimum of, 136
product inhibition in, 79
steroids and, 208

  ω-substituted fatty acids and, 405
  vitamin A and, 206
  zinc and, 178
Furacin, reduction of, 413
Fusarium solanum, pigment of, 422
Fusarubin, sulfate and, 420, 422
Fusiformis nigrescens, vitamin K and, 191

G

Gadidae, appendices pyloricae in, 375
Gadus callarias, uridine diphosphate glucuronic acid in, 448
Gadus luscus, neurohypophyseal hormones of, 306, 308
Galactose,
  enzyme induction by, 84
  glycoside formation and, 418
  intestinal absorption of, 349–350, 354
  lipid synthesis and, 164
β-Galactosidase,
  amount synthesized, 106
  induction of, 82, 85, 86, 89
  repressor of, 97, 98
β-Galactosides,
  permease, induction of, 85
Galago, tapetum lucidum of, 164, 189
Galleria,
  juvenile hormone in, 277, 279
  wax digestion by, 388
Galleria mellonella,
  acetylation in, 424
  ecdysone action in, 275
Gallionella, free energy of, 145
Gallium, requirement for, 186
Gallus domesticus, neurohypophyseal hormones of, 307
Gammexane, *see* Hexachlorocyclohexane
Gamones, inhibition of, 261
Gas,
  volcanic, composition of, 130
Gasterophilus,
  hemolymph, organic acids in, 141
  rhodanese in, 434
Gastric acid, chloride requirement and, 177
Gastric juice,
  endoproteinase in, 357–358
  lipase of, 358, 359
  milk clotting enzyme in, 358, 359
Gastric mill, absorption and, 356
Gastropods,
  acetylcholine in, 250
  food uptake by, 355
  juvenile hormone in, 280
  neurosecretion in, 261
  phagocytosis in, 380–381
Gelatin, spore germination and, 9
Gelatinase(s),
  bacterial, magnesium and, 176
Genetic material, metamorphosis hormone and, 237–239
Genlisea, prey capture by, 390
Gentiobiose, detoxification and, 416–419
Gentisic acid, formation of, 409
Geraniol, function of, 288
Gerbera jamesoni, glycosides in, 416, 418
Gerbil(s), aniline and, 407
Germination,
  altered enzyme activity and, 14–19
  endogenous reserves and, 8–10
  energy requirement for, 5–10
  initiation of, 5
  respiration and, 5–8
  seeds,
    electron transport and, 31
    endogenous reserves and, 31–36
    initiation of, 30–31
Giraffidae, stomach of, 361
Gizzard,
  contractions of, 356
  pH of, 370
  structure of, 367
Gladiolus, glycosides in, 417, 419
Glandectomy, amino acid levels and, 227–228
Globins, amphibian differentiation and, 56
Globulin(s), copper in, 180
Glomerella, glutathione requirement of, 153
Glossobalanus, iodide fixation by, 282–283
Glucagon, bird and, 332
Glucocorticoids, alkaline phosphatase and, 103
Glucosamine, chitin formation and, 229
Glucose,
  ascorbate synthesis from, 194

  detoxification and, 413, 415–417
  insect blood and, 141
  intestinal absorption of, 349–350, 354, 355, 373
  oxidation, sea urchin eggs and, 47
  phenylalanine synthesis and, 156
  phosphorylation, inhibition of, 78
  plant sap and, 136
  plasmodium formation and, 20
  seed germination and, 34
  slime mold fruiting and, 163
  sorocarp respiration and, 22
  spore germination and, 5, 10
  spores and, 15, 16
  sporulation and, 11–13, 18, 24–25, 27, 28
  total annual production of, 126
  water absorption and, 352, 353
Glucose dehydrogenase, sporulation and, 18
Glucose-6-phosphatase, amphibian differentiation and, 61
Glucose-1-phosphate,
  oxidation, sea urchin eggs and, 49
Glucose-6-phosphate,
  hexokinase inhibition by, 78, 79
  sea urchin eggs and, 47
Glucose-6-phosphate dehydrogenase,
  activation of, 104, 105
  inactive form of, 104
  sea urchin eggs and, 48–50, 65
α-Glucosidase, induction of, 89, 90
β-Glucosidase,
  induction of, 89
  occurrence of, 147
Glucuronate, crystalline style and, 384
β-Glucuronidase, detoxification and, 419
Glucuronides,
  detoxification and, 404, 413, 422, 442, 448
  development and, 445
  permeases, induction of, 85
Glucuronyltransferase, occurrence of, 420
Glutamate,
  adenylcobamide and, 201, 202
  arginine and, 154
  arginine-vasopressin and, 310–311
  arginine-vasotocin and, 312
  carnitine and, 205
  enzyme repression by, 102–103
  formation, 75, 150
    abiogenic, 131
  folic acid synthesis and, 200
  histidine degradation and, 156
  lysine biosynthesis and, 155
  β-melanocyte stimulating hormone and, 319, 323, 324, 327
  oxytocin and, 309
  pupal molt and, 227, 228, 229
  racemic, resolution of, 131
  requirement for, 159
  spore germination and, 9
  transport of, 351–352
  use in biosyntheses, 150
Glutamic dehydrogenase,
  differentiation and, 29–30
  zinc and, 178
Glutamic semialdehyde, proline and, 153
Glutaminase I, repression of, 102–103
Glutamine,
  detoxification and, 427, 431
  enzyme repression by, 102
  folic acid and, 200
  histidine synthesis and, 155
  importance of, 150
  insect blood and, 140
  larva and, 228, 229
  purine and pyrimidines and, 167
  requirement for, 150
Glutamine synthetase, product inhibition of, 78
Glutamyl transferase, repression of, 99, 102
Glutathione,
  DDT dehydrochlorinase and, 439
  detoxification and, 433
  electron transport and, 195
  formation of, 149, 150
  glycine formation and, 149
  metamorphosis and, 153
  spermidine and, 211
Glutathione reductase, insulin B chain and, 338
Glutathione synthetase, product inhibition of, 78–79
Glutelins, arginine and, 155
Glycera dibranchiata, juvenile hormone in, 280
Glycerol, insect blood and, 141

Glycerol dehydrogenase,
  inactive form of, 105
  induction of, 86
Glycerol phosphate, spore germination and, 9
α-Glycerophosphate dehydrogenase, metamorphosis and, 226
Glycinamide, oxytocin and, 309
Glycine,
  arginine-vasopressin and, 310–311
  arginine-vasotocin and, 312
  conjugations, development and, 445–446
  detoxification and, 426–432, 442
  formation, 149
    abiogenic, 131
  insulin and, 333, 336
  oxytocin and, 309
  pupal molt and, 227, 228, 229
  requirement for, 149, 158, 159, 162, 163, 203, 432
  serine and, 149, 151
  spore germination and, 7
  structural proteins and, 149
  threonine synthesis from, 151
  transport of, 351, 356
  use in biosyntheses, 149–150
Glycine N-acylase, detoxification and, 430–431
Glycine transamidinase, repression of, 100–101
Glycogen,
  amphibian differentiation and, 54
  hepatopancreas and, 382
  insulin and, 331, 338
  metamorphosis and, 231
  sea urchin egg development and, 41
  synthesis of, 162
    trehalose and, 231
  threonine and, 152
Glycolate,
  abiogenic formation of, 131
  serine requirement and, 151
Glycolate oxidase,
  activation of, 104
  formation of, 91
Glycolipids, formation of, 163
Glycolysis,
  intestinal absorption and, 353
  rate, control of, 79, 80
  sea urchin egg and, 47
Glycosides, detoxification and, 413–420
Glycuronconjugation, hormones and, 300
Glycylglycine dipeptidase,
  cobalt and, 184
  manganese and, 182
Glyoxylate, serine requirement and, 151
Glyoxylate cycle,
  control of, 81
  seed germination and, 35, 36, 38
Goat(s),
  acetylation in, 423
  hippuric acid in, 427
  phenothiazine and, 410
  rhodanese in, 434
Goldfish, glucuronides and, 414
Gomori substance, posterior pituitary and, 314
Gonads,
  copper in, 180
  hormones of, 300
Gonadostimulin, source of, 283
Gonadotropic hormone,
  action of, 279
  bioassay of, 279, 282
  formation, control of, 282
  physiological effects of, 282
Gonionemus murbachii, juvenile hormone in, 280
Gonocytes, neurosecretions and, 259–261
Goose, benzoic acid and, 429
Gorgonins,
  amino acids in, 160
  iodine and, 284
  occurrence of, 177
Grasshopper, diapausing, 225
Grit, gizzard and, 368
Guanidoacetate, enzyme repression by, 101
Guanidoacetate methylferase, enzyme repression and, 100, 101
Guanine,
  requirement for, 166
  tapetum lucidum and, 164
Guanophores, chromatotropins and, 263
Guanylate, requirement for, 166
Guinea pig(s),
  2-acetamidofluorene and, 407
  acetylation in, 423

amino acid transport in, 352
arginine requirement of, 154
ascorbate and, 194
enzyme induction in, 92
enzyme repression in, 102–103
glucuronides in, 414, 445
hippuric acid in, 427
ileum, acetylcholine analogs and, 253
inactive enzyme in, 104
mercapturic acids in, 432
naphthalene and, 408
sterol requirement of, 209
tryptophan requirement of, 157
uterus, oxytocic activity and, 302
Gymnosperms, enzyme induction in, 90
Gyptol, chemical nature of, 287
Gyrodinium cohnii, amino acid requirement of, 158

H

Hake,
pituitary powder, activity of, 314
Halides, requirement for, 176–178
Halobacterium halobium, pigment of, 138
Halogenobenzenes, detoxification of, 433
Halophiles, salt requirements of, 137
Hamster,
amino acid transport in, 351
mercapturic acids in, 432
Heart,
adrenaline effect on, 254
5-hydroxytryptamine and, 256
HeLa cells,
enzyme induction in, 95
enzyme repression in, 102
glutamine and, 150
sodium and, 174
Helix,
carbohydrate hydrolysis by, 147
chitinase of, 385–386
ethereal sulfates and, 422
heart, adrenaline and, 254
intestinal absorption in, 354
phagocytosis in, 381
phenols and, 418
Helix pomatia,
acetylcholine effect on, 250
cellulase in, 385–387
proteinase of, 384
sterols and, 209
vitamin A and, 207
Hematopoiesis, cobalt and, 185
Hematoxylin, neurosecretory cells and, 267
Heme, requirement for, 179
Hemerythrins, iron and, 179
Hemicellulose,
digestion of, 385
seed germination and, 32
Hemigrapsus, phosphate uptake by, 355
Hemimetabola,
differentiation in, 221, 222, 226
pars intercerebralis of, 267
Hemiptera,
ecdysone action in, 275
glucosides in, 415
juvenile hormone effect in, 278
Hemiselmis virescens, amino acid requirement of, 158
Hemocyanin, copper and, 180, 181
Hemoglobin,
formation, copper and, 181
iron and, 179
porphyrins and, 189
synthesis, repression of, 99
Hemolymph, amino acids in, 227
Hemophilus,
porphyrin and, 190
pyridine nucleotide and, 188
Hemophilus parainfluenzae, polyamine requirement of, 210–211
Hemosycotypin, zinc and, 178
Hemp,
seed, germination of, 32
Hen(s), *see also* Chick, Chicken, Fowl
benzoic acid and, 427–429
detoxification in, 404
ethereal sulfates in, 421
gizzard pH in, 370
methylation in, 425
phenylacetic acid and, 427
rhodanese in, 434
uterus, oxytocic activity and, 302
Hepatic necrosis, selenium and, 185
Hepatopancreas,
chitin digestion by, 386
copper in, 180
enzymes of, 384
food absorption and, 355

  function of, 381–382
  occurrence of, 381
  rhythmic secretion of, 385
  structure of, 381
Heptachlor, metabolism of, 411
Herbivores,
  digestion in, 366–367
  glycine conjugation in, 427
  intestinal pH in, 360
  methylation in, 426
  stomach contents of, 360
  symbiosis and, 389
Heron, gastric pH in, 359, 370
Herring, pancreas of, 374–375
Heterocycles, α-oxidation of, 411–412
Heterotrophs,
  free energy of, 146–148
  primitive, amino acids and, 158
Hexachlorocyclohexane,
  dehydrochlorination of, 437
  detoxification of, 433
n-Hexadecane, oxidation of, 146
Hexapeptide, melanotropic activity of, 329
Δ^2-Hexenol acetate, secretion of, 287
Δ^3-Hexenol-1, function of, 288
Hexobarbital, detoxification of, 405, 446
Hexokinase,
  product inhibition of, 78, 79
  sea urchin egg development and, 50
  spores and, 15, 65–66
Hexosamine, crystalline style and, 384
Hexose monophosphate shunt, regulation of, 81
Hickory, aluminum and, 186–187
Hindbrain, cholinesterase in, 59–60
Hippopotamus, vasopressin of, 312
Hippuric acid,
  detoxification and, 404
  formation of, 431
  glycine and, 150
  species forming, 427, 428
Hirudinea, neurosecretion in, 260
Hirudo medicinalis, adrenaline in, 254
Histamine,
  gastric secretion and, 370
  histidine and, 156
  pepsin secretion and, 373
Histidase, induction of, 86
Histidine,
  biosynthesis of, 155–156
  degradation of, 156
  hemolymph and, 227, 228
  morphogenesis and, 26–27
  requirement for, 155, 158, 159–163, 203
  synthesis,
    feedback inhibition of, 76, 77
    repression of, 96, 97
  transport of, 351, 352
Histones, arginine and, 155
Hog, *see also* Pig
  corticotropin of, 316, 320, 325–326
  insulin of, 336
  α-melanocyte-stimulating hormone of, 318–319, 321
  β-melanocyte-stimulating hormone of, 319, 322
  oxytocin of, 309–310
  pituitary, hormones of, 301, 304, 307
  posterior pituitary powder, activity of, 314
  vasopressin of, 311–312
Holocephali, digestive tract of, 376
Holocrine secretion, digestive enzymes and, 348
Holometabola,
  differentiation in, 221, 222
  pars intercerebralis of, 267
Holothuria,
  intestinal absorption in, 354–355
  muscle, adrenaline and, 254
Holothuria forskali, collagen of, 160
Holothurians, acetylcholine in, 249–250
Holothuroidea, juvenile hormone in, 281
Homarus,
  intestinal absorption in, 355
  juvenile hormone in, 280
Homocysteine, methionine and, 153
Homoserine, threonine synthesis from, 151
Homoserine kinase, feedback inhibition of, 77
Honey bee, *see* Bee
Honey guides, digestion of, 372
Hordeum vulgare, rhodanese in, 434
Hormones,
  classification of, 247–248
  digestive enzymes and, 348
  general considerations, 299–300

- invertebrate, general considerations, 246–247
- protein,
  - classification of, 300–302
  - synthesis of, 339–340

Horse,
- acetylation in, 423
- colon and cecum of, 365
- digestion in, 366
- food fermentation in, 360
- hippuric acid in, 427
- insulin of, 336
- α-melanocyte-stimulating hormone of, 319, 321
- β-melanocyte-stimulating hormone of, 323, 324
- neurohypophyseal hormones of, 307
- oxytocin of, 309–310
- posterior pituitary powder, activity of, 314
- rhodanese in, 434
- vasopressin of, 311

Housefly, *see also* Fly
- retinene in, 207

Human,
- acetylation in, 423–424
- amino acid requirements of, 163
- *p*-aminosalicylic acid and, 427
- arginine and, 154
- aromatic amino acid hydroxylation by, 445
- calcium requirement of, 169
- copper requirement of, 181
- corticotropin of, 320–321, 325
- enzyme induction in, 92
- enzyme repression in, 102
- feedback inhibition in, 78
- fetal, ethereal sulfates in, 444
- gastric pH in, 359
- glucuronides in, 445
- glutamine N-acylase in, 431
- hippuric acid in, 427
- histidine requirement of, 155
- inactive enzymes in, 104
- indoyl-3-acetic acid and, 427
- insulin of, 336
- iodine requirement of, 178
- iron deficiency in, 179–180
- manganese requirement of, 182
- α-melanocyte stimulating hormone of, 319
- β-melanocyte-stimulating hormone of, 319, 320, 322, 323, 324
- mercapturic acids in, 432
- methylation in, 426
- neurohypophyseal hormones of, 307
- oxytocin of, 309–310
- phenylacetic acid and, 427
- quinine oxidation in, 412
- rhodanese in, 434
- saliva, amylase in, 357
- stomach, endoproteinases in, 357
- thiamine deficiency in, 196
- tissues, amino acid requirements of, 163
- tocopherol and, 192
- uric acid in, 140
- vasopressin of, 311
- vitamin B_{12} requirement of, 202
- zinc deficiency in, 179

Hyalophora cecropia,
- amino acids in, 228, 230
- blood proteins of, 229
- brain hormone in, 267–268
- cytochrome b_5 in, 225
- cytochrome oxidase in, 224, 225
- diapause in, 269
- ecdysone action in, 275, 276
- ecdysone bioassay and, 272
- extracts, ecdysone in, 273
- juvenile hormone in, 277
- metamorphosis, respiration and, 224
- nucleic acids in, 232
- phosphate exchange in, 232
- trehalose in, 231

Hydra,
- copper requirement of, 180–181
- glutathione and, 195

Hydration, spore germination and, 5

Hydrazide, acetylation of, 423

Hydrocarbons,
- oxidation of, 146
- polycyclic, metabolism of, 407–408
- unsaturated, polyglycine and, 132

Hydrochloric acid,
- fish and, 376
- hexachlorocyclohexane and, 437
- secretion, 373
  - stimulation of, 370

Hydrogen,
  amino acid formation and, 130, 131
  oxidation of, 143–144
  photosynthesis and, 142–143
  primitive biosphere and, 129
Hydrogenase,
  activation of, 104
  algal, induction of, 88
  induction of, 86
  molybdenum and, 183
Hydrogen chloride, volcanic gas and, 130
Hydrogen fluoride, volcanic gas and, 130
Hydrogenomonas,
  free energy of, 143–144
  hydrogenase, induction of, 86, 104
Hydrogen sulfide,
  oxidation of, 144–145
  photosynthesis and, 142
  volcanic gas and, 130
Hydrolases,
  induction of, 83
  magnesium and, 176
Hydrolysis, detoxification and, 403–404, 434–436
Hydromineral regulation, neurohypophyseal hormones and, 315–316
Hydroosmotic activity, frog and, 303
Hydroquinone,
  detoxification of, 415
  formation, salt and, 138
  tanning and, 235
Hydrosulfite, hydrogenase and, 88
1(3)-Hydroxyacetamidofluorene, formation of, 407
3-Hydroxyanthranilic acid,
  detoxification of, 429, 430
  formation, insect and, 187
  vitamin B_6 and, 198
Hydroxyapatite, skeletons and, 168
p-Hydroxybenzaldehyde, detoxification of, 416
5-Hydroxybenzimidazole, vitamin B_{12} and, 201
m-Hydroxybenzoate, oxidation of, 409
α-Hydroxybutyrate, abiogenic formation of, 131
2-Hydroxy-4-chlorophenoxyacetic acid, formation of, 409
p-Hydroxycinnamic acid, methylation of, 426
7-Hydroxycoumarin, detoxification of, 415
3-Hydroxydecanoyl-L-serine, 151
6-Hydroxy-2,4-dichlorophenoxyacetic acid, formation of, 406
5-Hydroxyindoleacetic acid, excretion of, 157
Hydroxykynurenine, vitamin B_6 and, 198
Hydroxylysine, occurrence of, 154, 160
Hydroxynaphthoquinones, sulfate and, 420, 422
p-Hydroxyphenylpropionate, formation of, 235
Hydroxyproline,
  ascorbate and, 194
  formation of, 153–154
  glutamate and, 150
  larva and, 229
  occurrence of, 153, 154
  transport of, 351
p-Hydroxyphenylpyruvate, tanning and, 235
p-Hydroxyphenylpyruvate oxidase, inactive form of, 104
8-Hydroxyquinoline, detoxification of, 414, 415
11-β-Hydroxysteroid dehydrogenase, induction of, 92–93
β-Hydroxy-γ-trimethylaminobutyric acid, *see* Carnitine
5-Hydroxytryptamine, 270
  biosynthesis of, 256
  invertebrates and, 255–256
5-Hydroxytryptophan, derivatives of, 157–158
Hydroxytyramine, occurrence of, 254
Hydrozoa, juvenile hormone in, 280
Hyla cinerea, acetylation in, 424
Hylemya antiqua, lipoate and, 199
Hymenolepis diminuta, vitamin B_6 and, 198
Hymenoptera,
  ecdysone action in, 275
  5-hydroxytryptamine in, 255
  juvenile hormone in, 280
Hypobranchial gland,
  5-hydroxytryptamine in, 255, 256
  secretion of, 248

Hypochytriales, lysine biosynthesis by, 124
Hypoglycemic action, insulin and, 331, 332
Hypophysis, subneural gland of, 283
Hypothalamus, neurohypophyseal hormones and, 313–314

I

Imidazole, histidine requirement and, 155
Imidazole acetic acid, histidine degradation and, 156
Imidazole acrylic acid, histidine requirement and, 155
Iminoaceticpropionic acid, abiogenic formation of, 131
Iminodiacetic acid, abiogenic formation of, 131
2-Iminothiazolidine-4-carboxylic acid, cyanide and, 433
Indigo, formation of, 422
Indole,
  product inhibition by, 79
  tryptophan synthesis and, 157
  tryptophan synthetase and, 95
Indoleacetic acid,
  metabolism, riboflavin and, 189
  synthesis, zinc and, 178
  tryptophan and, 157
Indoleacetic acid oxidase, inactive form of, 105
Indoxylsulfate, occurrence of, 422
Indoyl-3-acetic acid, glutamine and, 427
Injury,
  glycogen metabolism in, 231
  respiration and, 226, 230
Inositides, synthesis of, 164
Inositol,
  function of, 210
  lipid synthesis and, 164
  metabolism, manganese and, 182
  microorganisms and, 170
  requirement for, 173, 210
Insects,
  acetylation in, 424, 425
  acetylcholine and, 249
  adrenaline in, 254
  amino acid requirements of, 160–161
  arginine and, 154
  aromatic acids and, 429, 430, 432
  carbohydrate in, 231
  carnitine and, 205
  carotenoids in, 207
  chitinase in, 386
  choline and, 204
  chromatotropins in, 266
  comparative nutrition of, 122
  DDT metabolism in, 438–441
  detoxification in, 404
  diapausing, cyanide and, 10
  enzyme secretion in, 385
  ethereal sulfates in, 420, 421, 448
  extraintestinal digestion in, 388–389
  eye pigments, tryptophan and, 158
  "fungus gardens" of, 389
  glucoside formation in, 414–415
  growth of, 222
  gut of, 382
  hemolymph, amino acids in, 227
  hepatopancreas and, 381
  hexachlorocyclohexane and, 437
  inactive enzyme in, 104
  internal pH of, 138
  intestinal absorption in, 355–356
  juvenile hormone in, 280
  lipids in, 231
  magnesium requirements of, 176
  malaoxon and, 443
  mercapturic acids in, 433
  methylation in, 425, 426
  neurosecretion in, 265–270
  nicotinamide and, 187
  nitro groups and, 413
  nonneural hormones of, 272–282
  osmotic balance in, 138, 140–141, 212
  pantothenate and, 199
  phagocytosis and, 381
  phosphoramides and, 411
  phosphorothioates and, 410
  potassium requirement of, 174–175
  protein requirement of, 149
  pterins in, 200
  purine and pyrimidine requirements of, 167
  pyrethrins and, 434–434, 441
  salicin and, 405
  serine requirement of, 151
  social, pheromones of, 287–288
  sterols and, 209

  Systox and, 410
  tocopherol and, 192
  tyrosine metabolism in, 157
  venom, serotonin in, 157–158
  wood utilization by, 147, 387
  zinc requirement of, 178
Insecticides, mercapturic acids and, 433
Insectivores, chitinase in, 361
Insectoverdin, corpora allata and, 234
Insulin(s),
  biological properties of, 331–332
  chains,
    activity of, 338
    determination of number, 333
    structure of, 334
    separation of, 333–334
    reassociation of, 337–339
  disulfide bridges of, 334, 335
  general considerations, 330–331
  modifications of, 314
  purification of, 332–333
  reduced, activity of, 338
  structure, biological activity and, 336–339
  zinc and, 178
Intermedins, mammals and, 301
Intestine,
  bird, pH of, 370
  movements of, 356
  nitrogen uptake in, 364
  phagocytosis in, 378
  pH of, 360
  proteins, differentiation and, 56
  surface of, 349
Intrinsic factor, vitamin B_{12} and, 203
Invertebrates,
  arginine requirement of, 154–155
  cations in, 138
  digestion in, 377–390
  emulsifiers in, 384
  ethereal sulfates in, 420
  fresh water, blood osmotic pressure of, 139
  hormones, general considerations, 246–247
  intestinal absorption in, 354–356
  marine, blood osmotic pressure of, 139
  nicotinamide and, 188
  osmotically active substances in, 212
  skeletal protein, glycine and, 149
  uric acid in, 140
  vitamin A in, 206, 207
Iodide,
  fixation,
    Enteropneustes and, 283
    invertebrates and, 284
    tunicates and, 283–284
Iodine, requirement for, 177–178
Iodoacetate, monosaccharide absorption and, 350, 351
Iodonin, occurrence of, 177
Iodotyrosine, occurrence of, 160
Iridomyrmex detectus, pheromone of, 287
Iris japonica, glycosides in, 416
Iron,
  enzyme induction and, 94–95
  function of, 171
  molybdoflavoproteins and, 183
  plants and, 137
  requirement for, 128, 179–180
  transport, silicon and, 167
  uptake of, 355
Iron bacteria, free energy of, 145
Isoamyl acetate, function of, 288
Isocaprolate, leucine synthesis and, 152
Isocitratase,
  induction of, 89
  repression of, 96, 99
  succinate and, 81
Isocitrate dehydrogenase,
  activation of, 104
  inactivation, differentiation and, 28–29
  manganese and, 181–182
Isoleucine,
  arginine-vasotocin and, 312
  biosynthesis of, 152
  feedback inhibition and, 77
  insulin and, 336
  oxytocin and, 309
  requirement for, 152, 159–163
  threonine deaminase and, 76
  threonine synthesis and, 151
  transport of, 351
Isomerases, induction of, 83
Isonicotinic hydrazide,
  acetylation of, 423–424
  pyridoxal kinase and, 197
Isopoda,
  androgenic gland in, 271
  chitinase of, 385
Isoprene units, leucine and, 152

Isoptera, ecdysone action in, 275
Isotocin, isolation of, 308
Isotopic competition, feedback inhibition and, 75

J

Juvenile hormone,
  bioassay of, 277
  distribution of, 278, 280–281
  formation and function of, 277
  insect development and, 265–266, 269
  molting and, 276
  physiological effects of, 278–279
  purification and chemistry of, 277–278

K

Kalanchoe punctata, glycosides in, 416
Kalotermes flavicollis, ecdysone action in, 275
Kelthane, formation of, 440, 441
Keratin,
  digestion of, 387–388
  gizzard and, 367
  glycine and, 149
Kerkring, folds of, 349
Kestrel, gizzard pH in, 370
Keto acid,
  prebiotic formation of, 131
  utilization of, 152
  manganese and, 182
4-Ketoamyltrimethylammonium ions, acetylcholine and, 252–253
α-Ketobutyrate,
  isoleucine synthesis and, 152
  source of, 153
  thiamine and, 195
  threonine and, 152
α-Ketoglutarate,
  germinated seeds and, 37
  glutamate formation from, 150
  insect blood and, 141
  lipoate and, 199
  thiamine and, 195
α-Ketoisovalerate,
  leucine synthesis and, 152
  pantothenate and, 198
  valine synthesis and, 152
α-Keto-γ-methiolbutyrate, utilization of, 153
Ketoproline, occurrence of, 154
α-Ketovalerate, thiamine and, 195
Kidney,
  amino acid conjugation in, 430–431
  detoxification and, 447–448
  oxygen consumption of, 148
  rhodanese in, 434
Kinases(s),
  enzyme activation by, 105
  induction of, 83
Klebsiella pneumoniae, iron requirement of, 179
Krebs cycle, *see* Citric acid cycle
Krebs-Henseleit cycle, differentiation and, 61
Krypton, primitive biosphere and, 129
Kynureninase, induction of, 89, 90
Kynurenine, nicotinamide synthesis and, 187

L

Labyrinthula vitellina, sterol requirement of, 209
Lacerta viridis,
  acetylation in, 424
  aromatic acids and, 429
  digestive secretions in, 373
  ethereal sulfates in, 421
  glucuronides in, 414
Lactalbumin, amino acids in, 159
Lactose, bird and, 371
Lactate,
  abiogenic formation of, 131
  hydrogen utilization and, 144
  insect blood and, 141
  ruminant and, 361, 365
  sea urchin eggs and, 47
  seed germination and, 31
  spore germination and, 5
Lactate dehydrogenase, zinc and, 178
Lactic acid bacteria, Pasteur effect in, 80, 81
L-Lactic acid cytochrome c reductase, induction of, 19
L-Lactic dehydrogenase,
  activation of, 104
  formation of, 90
Lactobacilli, linoleic acid and, 193
Lactobacillus,
  thymine assay and, 165
  uracil assay by, 165

Lactobacillus acidophilus,
  deoxyribosides and, 165
  vitamin B_6 and, 197
Lactobacillus arabinosus,
  amino acid requirement of, 159
  folic acid synthesis by, 201
  malic enzyme, induction of, 87
  manganese and, 182
  nicotinamide and, 188
  vitamin B_6 and, 197
Lactobacillus bifidus,
  growth, vitamin K and, 191
Lactobacillus brevis,
  cytosine assay and, 165
  uracil assay by, 165
Lactobacillus bulgaricus,
  orotic acid and, 165
  ureidosuccinate and, 165
Lactobacillus casei,
  orotic acid and, 166
  polyamine requirement of, 210–211
  rhizopterin and, 200
  strepogenin and, 211
  vitamin B_6 and, 197
Lactobacillus helveticus, vitamin B_6 and, 197
Lactobacillus heterohiochii, mevalonate and, 206
Lactobacillus lactis,
  thymidine assay and, 165
  vitamin B_{12} and, 202
Lactobacillus leichmannii,
  thymidine assay by, 165
  vitamin B_{12} and, 202–203
Lactobacillus plantarum,
  amino acid requirements of, 159
  growth, adenine and, 165
Lactobacteriaceae, enzyme induction in, 84, 87
Lactose, intestinal pH and, 370
Lagenaria leudantha, glycosides in, 417
Lamellibranchs,
  acetylcholine in, 250
  crystalline style of, 384
  digestive enzymes of, 384
  food uptake by, 355
  neurosecretion in, 260–261
  phagocytosis in, 380, 381, 384
Lamprey, vitamin A of, 206
Lasioderma serricorne, vitamin B_6 and, 198
Lead, accumulation of, 187
Leander aspersus, chromatotropins and, 265
Leaves, oxygen consumption of, 148
Lebistes, tocopherol and, 192
Lecithins,
  serine and, 151
  synthesis of, 163
Leishmania, porphyrin and, 190
Lens, riboflavin in, 171
Lentils, glycosides in, 416
Lentinus lepideus, methylation in, 426
Lepidoptera,
  differentiation, 222
    respiration and, 224
  ecdysone action in, 275
  egg ripening in, 279
  extracts, ecdysone in, 273
  glucosides in, 415
  juvenile hormone in, 278, 280
Lepisma,
  ethereal sulfates in, 421
  7-hydroxycoumarin and, 415
Leprotene, vitamin A and, 206
Leptinotarsa decemlineata, diapause in, 269
Leptodora,
  heart, adrenaline and, 254
Leptostraca, androgenic gland in, 271
Leptosynapta inhaerens, juvenile hormone in, 281
Leptothrix, free energy of, 145
Leucine,
  corticotropin and, 321
  isoprene units and, 152
  oxytocin and, 309
  requirement for, 152, 159–163
  sea urchin egg development and, 44–45
Leucine aminopeptidase,
  corticotropin and, 330
  insulin activity and, 337
  manganese and, 182
  neurohypophyseal hormones and, 312
Leucocytes,
  bacterial intake by, 378
  zinc in, 178

Leuconostoc mesenteroides,
  amino acid requirement of, 159
  malic enzyme, induction of, 87
  nicotinamide and, 188
  thymidine assay by, 165
Leucophaea,
  DDT metabolism in, 440, 441
  gonadotropic hormone in, 279
Leucovorin, *see* Folic acid
Levantina hierosolyma, cellulase in, 386–387
Lichenase, fish and, 376
Lichenin, digestion of, 385
Life,
  terrestrial, origin of, 128–129
Lignin,
  degradation, induction of, 89
  digestion of, 372, 387
  tyrosine and, 156
Limacidae, sex hormone in, 271
Limnoria lignorum, cellulase in, 386, 387
Limulus,
  heart, adrenaline and, 254
  juvenile hormone and, 280
Linoleic acid,
  function of, 193
  requirement for, 193–194
Linolenic acid, function of, 193
Lipase,
  crystalline style and, 384
  detoxification and, 434
  gastric, 358, 359
  loss of, 389
  seed germination and, 32, 36
  vertebrate and invertebrate, 383
  wax and, 388
Lipid,
  egg, 55
  enzyme stability and, 17
  epicuticle and, 234
  metabolism, metamorphosis and, 231
  sea urchin egg development and, 41
  synthesis, 163
    insulin and, 331–332, 338
Lipoic acid,
  function of, 199
  microorganisms and, 170
  pantothenate and, 198
  requirement for, 199
  sulfite oxidation and, 143
  thiamine and, 195
Lipopolysaccharides,
  formation of, 163
  monosaccharides in, 162
Lipoprotein, synthesis of, 163
Lipoprotein lipase,
  calcium and, 175
  magnesium and, 176
  manganese and, 182
Lipoxidase(s), essential fatty acids and, 193
Lithium,
  ion antagonism and, 186
  sea urchin egg development and, 45–46
  skeleton formation and, 168
Liver,
  amino acid conjugation in, 430–431
  copper in, 180
  enzyme induction in, 93
  ergothionine in, 156
  fat, threonine and, 152
  feedback inhibition in, 78
  glucosyl transferase and, 420
  oils, iodine in, 177
  product inhibition in, 78, 79
  rhodanese in, 434
Lizard, aromatic acids and, 430
Locust(s),
  amino acid absorption in, 355
  chlorobenzene and, 406, 407, 436
  β-carotene oxidation by, 405
  ecdysone in, 273
  mercapturic acids in, 432–433
  reductive dehalogenation in, 436
Locusta,
  ethereal sulfates in, 421
  glucosides in, 415
  juvenile hormone effect in, 279
  pigment of, 234
Locusta migratoria,
  acetylation in, 424
  aromatic acids and, 429
  histogenesis in, 230
Loligo, epistellary body of, 271
Lombricine, 160
  serine and, 151
Loxostege sticticalis, essential fatty acids and, 193

Lucilia,
  collagenase of, 388
  metamorphosis, cystine and, 152
  methylation and, 426
Lumbricus terrestris,
  chitinase in, 386
  collagen of, 160
  juvenile hormone in, 280
Lycastis, neurosecretion in, 259
Lycopersicon esculentum, glycosides in, 417
Lycosa, ethereal sulfates in, 421
Lyctus, wood utilization by, 387
Lysine,
  aminobenzoic acid and, 431
  aspartate and, 150
  biosynthesis, 155
    alternate pathways of, 123–124
  cell walls and, 155
  β-melanocyte-stimulating hormone and, 319, 320, 323, 324
  pupa and, 227, 228
  requirement for, 155, 159–163
  sclerotization and, 234
Lysine decarboxylase, induction of, 86
Lysine-vasopressin,
  activities, species and, 305
  chemical synthesis of, 312
  occurrence of, 307
  structure of, 311–312
Lysozyme,
  lysis, spermine and, 211
Lysuric acid, formation of, 431

M

Macrotoma palmata, wood utilization by, 387
Magnesium,
  insect blood and, 140
  morphogenesis and, 27
  osmotic balance and, 138, 139
  oxytocic activity and, 303
  plants and, 137
  requirement for, 175–176
  sodium requirement and, 174
  surface water, origin of, 130
Magnesium fluorophosphate, fluoride toxicity and, 177
Maize, *see also* Corn
  germination of, 32
  glycosides in, 416
  iodine and, 177
Malacosoma americana, DDT metabolism in, 440
Malacostraca,
  juvenile hormone in, 280
  molting gland in, 271
  neurosecretion in, 262–265
Malaoxon, toxicity of, 443
Malate,
  aspartate formation from, 130
  insect blood and, 141
Malate synthetase,
  induction of, 89
  repression of, 99
Malathion, toxicity of, 443
Maleic hydrazide, detoxification of, 417, 418
Malic dehydrogenase,
  manganese and, 181–182
  metamorphosis and, 226
Malic enzyme, induction of, 87
Malignant carcinoid, hydroxyindoleacetate excretion in, 157
Mallophaga, keratin digestion by, 388
Mallotus japonicus, glycosides in, 416
Maltase,
  induction of, 90, 93
  localization of, 353
Maltose,
  absorption of, 373
  seed germination and, 32–33
Maltozymase, ultraviolet and, 19
Malus pumilia, glycosides in, 417
Malus sylvestris, glycosides in, 416
Mammals,
  acetylation in, 425
  aldrin and, 411
  amino acid requirement of, 162–163
  p-aminobenzoate deficiency in, 201
  blood osmotic pressure of, 139
  chloramphenicol and, 436
  choline and, 204
  colchicine and, 441
  comparative nutrition of, 122
  digestive enzyme secretion in, 348–349
  ergothionine in, 156
  ethereal sulfates in, 420
  feedback inhibition in, 78

fetal, glucuronides and, 445
fluorophosphonates and, 436
hemoglobin, oxygen and, 99
hexachlorocyclohexane and, 437
inactive enzymes in, 104
insulin action in, 331–332
juvenile hormone in, 281
magnesium requirement of, 176
malaoxon and, 443
melanocorticotropic hormones and, 317
melanocyte-stimulating hormone in, 316, 330
mercapturic acids in, 433
methoxychlor and, 438
methylation in, 425, 426
naphthalenes and, 407–408
neurohypophyseal hormone,
  activity in, 302–303
  purification of, 304–307
nitro groups and, 413
nonplacental, vasopressin of, 307
phosphorothioates and, 410
posterior pituitary powder, activity of, 314
product inhibition in, 79
protein requirement of, 159
reductive dehalogenation by, 436
thioether oxidation by, 409
threonine aldolase of, 151
tissues, glutamine requirement of, 150
tocopherol and, 192
ubiquinone of, 190
vitamin A requirement of, 208
vitamin B_3 and, 198
Mammary gland, calcium and, 175
Manganese,
  chelation of, 182
  glycine formation and, 149
  magnesium requirement and, 176
  oxidation, free energy and, 145
  requirement for, 128, 181–183
  spore germination and, 6–7, 9, 65
  sporulation and, 14
Manganese sulfate, seed germination and, 31
Mannose,
  insect blood and, 141
  intestinal absorption of, 349–350
Mars, habitability of, 134–135
Marsupials,
  digestion in, 366–367
  glucuronides in, 414
Maya squinado, chitinase in, 386
Medicago sativa, rhodanese and, 434
Melanin, tyrosine and, 156
Melanocorticotropic hormones,
  biological properties of, 317–318
  general considerations, 316–317
  purification of, 318–321
  structure, biological activity and, 327–330
Melanocytes, corticotropin and, 318
Melanocyte-stimulating hormones, derivation of, 301
α-Melanocyte-stimulating hormone,
  chemical synthesis of, 321–322
  corticotropin and, 325, 327
  purification of, 318–319
  structure of, 321
β-Melanocyte-stimulating hormone,
  chemical synthesis of, 324–325
  purification of, 319
  structure of, 322–324
Melanophores,
  chromatotropins and, 263
  neurohormone and, 270
Melanoplus differentialis, DDT metabolism in, 440
Melanoplus femur rubrum, DDT metabolism in, 440
Melasoma, salicin and, 405
Menadione, requirement for, 191
Menstruation, iron requirement and, 180
Menthol, glucuronide of, 414
Menthol glucuronide, β-glucuronidase and, 419
β-Mercaptoethylamine, coenzyme A and, 198
Mercapturic acids, formation of, 432
Mercury, habitability of, 134
Merluccius merluccius, neurohypophyseal hormones of, 308
Merocrine secretion, digestive enzymes and, 348
Merostomata, juvenile hormone and, 280
Mesaconate, formation of, 202
Metabolic controls, types of, 74

Metals,
  primitive biosphere and, 130
  vitamin B_6 and, 197
Metamorphosis,
  amino acid and protein metabolism in, 227–231
  carbohydrate metabolism and, 231–232
  conclusions regarding, 239–240
  cystine and, 152
  hormonal control of, 265–266
  hormone, genetic material and, 237–239
  lipid metabolism and, 231–232
  morphology and physiology of, 222–223
  nucleic acid metabolism and, 232
  phosphorus metabolism and, 232
  pigment synthesis and, 233–234
  respiration and, 223–227
  sclerotization and, 234–237
  specific metabolic pathways and, 232–237
Metaphyta, enzyme induction in, 90–91
Metazoa,
  enzyme induction in, 91–95
  enzyme repression in, 99–103
Methane,
  amino acid formation from, 130
  formation of, 144
  marsupial digestion and, 367
  oxidation of, 146
  primitive biosphere and, 130
  rumen and, 362
  volcanic gas and, 130
Methanomonas,
  free energy of, 146
  hydrogen utilization by, 144
Methionine,
  p-aminobenzoate and, 201
  choline and, 203, 204
  cysteine synthesis and, 152
  pupa and, 227
  requirement for, 153, 159–163
  sea urchin egg development and, 43–44
  selenium and, 185
  serine and, 151
  slime mold differentiation and, 22, 24–26
  spermine synthesis and, 211
  spore germination and, 9
  sporulation and, 14
  transport of, 351
  vitamin B_{12} and, 189
Methionine synthetase, repression of, 95, 96
Methoxychlor, metabolism of, 438
2-Methyladenine, vitamin B_{12} and, 201
N-Methylalanine, abiogenic formation of, 131
β-Methylaspartate,
  formation of, 201, 202
  function of, 202
Methylation, detoxification and, 403–404, 425–427
Methylene azure, oxidation of, 409
Methylene blue,
  oxidation of, 409
  tocopherol deficiency and, 192
  vitamin E and, 122
Methyl groups, methionine and, 153
Δ^2-Methylheptenone, function of, 287
Methyl nicotinamide, excretion of, 426
N-Methyl pyridinium hydroxide, occurrence of, 425–426
Methylthioaniline, oxidation of, 409
5-Methyltryptophan,
  enzyme derepression and, 96
  enzyme induction and, 82
Methylurea, abiogenic formation of, 131
Metridium senile, juvenile hormone and, 280
Mevalonate,
  carotenoid synthesis and, 206
  cholesterol synthesis from, 101
  isoprenoid compounds and, 191
  leucine and, 152
  steroids and, 208
Miastor, extraintestinal digestion in, 389
Microciona prolifera, juvenile hormone and, 280
Micrococcaceae, enzyme induction in, 84, 86
Micrococcus, linoleic acid and, 193
Micrococcus denitrificans,
  enzyme induction in, 84
  enzyme repression in, 96
  hydrogen utilization by, 144

Micrococcus glutamicus, feedback inhibition in, 77
Micrococcus halodenitrificans, salt requirement of, 137
Micrococcus pyogenes,
  penicillinase, induction of, 86
Micromonospora, juvenile hormone in, 281
Microorganisms, *see also* Bacteria, Fungi, Molds, Protista, Protozoa, Yeast
  chloramphenicol and, 436
  drug resistance in, 404
  fluorophosphonates and, 436
  glutathione and, 195
  juvenile hormone in, 281
  nitro groups and, 413
  soil, aromatic compounds and, 407–409
  Systox and, 410
Microsomes,
  detoxification by, 410–412, 431, 447
    development and, 446–447
  histogenesis and, 230
Microvilli, intestinal surface and, 349
Micrura caeca, juvenile hormone in, 280
Midbrain, cholinesterase in, 59–60
Milk,
  clothing, calcium and, 175
  copper in, 181
  digestion of, 359
  dried, intestinal pH and, 370
  gastric lipase and, 359
Milk-ejecting activity, mammals and, 303, 305
Mineral crystals, asymmetric syntheses and, 131
Mineral deposits,
  formation, requirements for, 167–169, 213
Mites, toxicant for, 441
Mitochondria,
  aromatic acids and, 431
  detoxification and, 410
  ecdysone and, 238
  ectoderm and, 45–47
  metamorphosis and, 224–225, 230
  rhodanese in, 434
  sea urchin egg development and, 44, 50–51
  seed germination and, 37–38
  spermidine and, 211
  vitamin K and, 191
Molds, *see also* Fungi, Microorganisms
  amino acids and, 158
  ethereal sulfates in, 420, 445
  lysine synthesis by, 155
  methylation in, 426
  sterols and, 208
  thiamine and, 165
Mollusks,
  acetylcholine in, 249, 250
  blood pigment, zinc and, 178
  calcium requirement of, 125
  copper in, 180, 181
  enzyme repression in, 99
  ethereal sulfates in, 420, 422, 445
  glycosidases in, 418
  hepatopancreas in, 381
  5-hydroxytryptamine in, 255, 256
  iodine fixation by, 284
  intestinal absorption in, 355
  juvenile hormone in, 280
  neurosecretion in, 260–261
  Pasteur effect in, 80
  punicin of, 177
  sex hormones in, 270–271
  skeletons of, 168
  vitamin A and, 207, 208
Molting,
  hormonal control of, 265–266, 275–276, 290
  metabolism and, 226
Molting hormone, ecdysone and, 271
Molybdenum,
  copper deficiency and, 181
  requirement for, 183–184
Molybdoflavoprotein, 183
  chemoautotrophs and, 143
Monilinia fructicola, dimethoxynaphthalene and, 435
Monkey,
  acetylation in, 423, 424
  hippuric acid in, 427
  α-melanocyte-stimulating hormone of, 319, 321
  β-melanocyte-stimulating hormone of, 319, 323, 324
  rhodanese in, 434
  tissues, amino acid requirements of, 149, 163

  vasopressin of, 307
  xanthopterin and, 200
Monoaminoadipic acid, lysine biosynthesis and, 124
Monocotyledons, enzyme induction and, 90
Monoglycerides, fat absorption and, 351
3-Monoiodotyrosine, tunicates and, 284, 285
Monomethylethanolamine, 204
Monosaccharides, requirement for, 141, 146
Moon, contamination of, 135
Morphogenesis,
  biochemistry, general considerations, 1–4
  "limiting step" in, 26–27
Morphology,
  bacterial, carbon source and, 145
Mosquito,
  benzoic acid and, 430
  gonadotropic hormone in, 279
  larvae,
    cystine and, 152–153
    glycine and, 149
Moth, sex attractant of, 286
Mottle leaf disease, zinc and, 178
Mouse,
  acethion and, 444
  acetylation in, 423
  amino acid requirement of, 162
  p-aminosalicylic acid and, 427
  enzyme induction in, 92
  glucuronides in, 444, 445
  growth requirements of, 170
  hexobarbital and, 405
  hippuric acid in, 427
  insulin assay in, 331
  intestine, phagocytosis in, 381
  L strain cells,
    glutamine and, 150
    sodium and, 174
  malaoxon and, 443
  mercapturic acids, in, 432
  methylthioaniline and, 409
  organs, enzymes in, 2
  strepogenin and, 211
  thiamine deficiency in, 196
  zinc requirement of, 178–179
Mouth,
  vertebrate, role in digestion, 357
Mucin,
  crystalline style and, 384
  peritrophic membrane and, 382
  salivary, function of, 357
Muconic acids, formation of, 409
Mucoprotein, formation of, 163
Mucor,
  methylation by, 426
  thiamine and, 195-196
Mucorales, lysine biosynthesis by, 124
Mullet, rhodanese in, 434
Murex,
  iron uptake by, 355
  neurohumor of, 248
Murex brandaris, prochromogen of, 422
Murex trunculus,
  5-hydroxytryptamine in, 255, 256
  prochromogen of, 422
Musca,
  acetylation in, 425
  aromatic acids and, 429
  metamorphosis, respiration and, 224
  succinic dehydrogenase in, 226
Musca domestica,
  DDT metabolism in, 439, 440
  nitrogen distribution in, 228
Musca vicina, amino acid requirement of, 161
Muscle,
  acetylcholine in, 249
  adrenaline effect on, 254
  glycogen, insulin and, 331
  proteins, differentiation and, 56
  skeletal, oxygen consumption of, 148
  smooth, 5-hydroxytryptamine and, 255
  striated, acetylcholine and, 251
Muscular dystrophy, tocopherol and, 192
Mussel,
  hippuric acid in, 430
  N-methyl pyridinium hydroxide in, 426
  phenols and, 414
Mya arenaria, juvenile hormone and, 280
Mycobacteriaceae, enzyme induction in, 84
Mycobacterium paratuberculosis,
  growth, vitamins K and, 191
Mycobacterium phlei, vitamin A and, 206

Mycobacterium tuberculosis, vitamin K of, 191
Myoglobin, iron and, 179
Myoneural synapses, acetylcholine and, 251
Myosin,
  calcium and, 175
  cardiac, differentiation and, 57
Myotropic activity, sources of, 269
Mytilus,
  5-hydroxytryptamine in, 256
  muscle, adrenaline and, 255
Myxamebas, permeability of, 24–25
Myxoxanthin, vitamin A and, 206

N

Naphthalene,
  metabolism of, 407, 432–433
  oxidation, calcium and, 175
α-Naphthol, detoxification of, 414
2-Naphthol phenolphthalein, detoxification of, 421
β-Naphthoxyacetic acid, metabolism of, 409
β-Naphthylamine,
  oxidation of, 407
  sulfate and, 420, 422
  tumors and, 407
Naphthylthiourea, toxicity of, 410
Nassanoff gland, pheromone of, 288
Natriferic activity, frog skin and, 304, 305, 316
Natrix natrix, aromatic acids and, 429
Neisseria gonorrheae,
  amino acid requirement of, 159
  thiamine and, 196
Neisseria perflava, polyamine requirement of, 210–211
Nematocysts, 5-hydroxytryptamine in, 255
Nematodes,
  gut of, 389
  pantothenate and, 199
  parasitic, Pasteur effect in, 80–81
  vitamin B_6 and, 198
Nemertinea, neurosecretion in, 257
Neodiprion lecontei, juvenile hormone in, 280
Neoplasms, folic acid analogs and, 200
Neotenin, metamorphosis and, 223
Nepenthes, proteolysis by, 390
Nephridium, neurosecretions and, 260
Nephrops,
  amylase, temperature and, 361
  intestinal absorption in, 355
Nephthys, neurosecretion in, 258, 259
Nereidia, neurosecretion in, 258, 259
Nereis virens, juvenile hormone in, 280
Nerve,
  digestive enzymes and, 348
  cells, acetylcholine in, 251
  centers, 5-hydroxytryptamine in, 255
  impulse, calcium and, 175
Nettle(s), thiocyanate in, 434
Neural tissue,
  differentiation, cholinesterase and, 59
Neurohumors, chemical nature of, 248
Neurohypophyseal hormones,
  action,
    in amphibia, 303–304
    in birds, 303
    in fish, 304
    in mammals, 302–303
    in reptiles, 303
  biological properties of, 302–304, 313
  biosynthesis of, 313–314
  chemistry of, 308–313
  evolution and, 313–316
  purification,
    batrachians, 307–308
    bird, 307
    fish, 308
    mammals, 304–307
Neurophysin,
  Gomori substance and, 314
  isolation of, 307
Neuroptera, ecdysone action in, 275
Neurosecretion, products of, 256–270
Neurosecretory cells,
  activity, control of, 268–269
  other hormones of, 269–270
Neurospora,
  arginine requiring, 154
  enzyme induction in, 90
  enzyme repression in, 98, 99
  ethereal sulfates and, 422
  folic acid and, 201
  glutamate and aspartate and, 150
  nicotinamide and, 188
  pyrimidines and, 166

- serine requiring, 151
- spores, germination of, 18
- threonine synthesis by, 151, 152
- tryptophan synthesis in, 157

Neurospora crassa,
- adenine assay and, 165
- choline and, 204
- cytidine assay by, 165
- feedback inhibition in, 77
- uridine assay by, 165

Nickel, cell growth and, 186

Nicotiana rustica, glycosides in, 417

Nicotiana sanderae, glycosides in, 417

Nicotinamide,
- biosynthesis of, 187–188
- detoxification of, 427
- electron transport and, 187–188
- requirement for, 187–188
- vitamin B_6 and, 198

Nicotine, oxidation of, 412

Nicotinic acid, 164
- detoxification of, 426, 429
- microorganisms and, 170
- occurrence of, 187
- tryptophan and, 158

Nicotinic acid mononucleotide, product inhibition by, 79

Nicotinic acid mononucleotide pyrophosphorylase, inhibition of, 79

Nicotinuric acid, formation of, 427, 430

Ninhydrin, chromatotropin and, 264

Nitrate,
- hydrogen and, 144
- methane oxidation and, 146
- reduction of, 144
- seed germination and, 36

Nitrate reductase,
- induction of, 89, 91
- molybdenum and, 183

Nitrification,
- calcium and, 175
- copper and, 180

Nitrite, oxidation of, 143, 144

Nitrite oxidase, molybdenum and, 183

Nitrobacter,
- free energy of, 144
- molybdenum and, 183

Nitrobacteriaceae,
- enzyme induction in, 84, 86
- free energy of, 144

Nitrobenzoate,
- conjugation of, 429
- oxidation of, 409
- reduction of, 413, 446

Nitrogen,
- balance, maintenance of, 127
- fixation,
  - calcium and, 175
  - iron and, 179
  - magnesium and, 176
  - molybdenum and, 183
  - vanadium and, 185
- Mars and, 134
- sea urchin egg development and, 41
- storage, plants and, 150
- Venus and, 134
- volcanic gas and, 130

Nitro groups, reduction of, 413

p-Nitrophenol, detoxification of, 414, 421

Nitroreductase,
- development and, 446
- nature of, 413

Nitrosomonas,
- calcium and, 175
- free energy of, 144

Nocardia, naphthalene and, 408

Nocardia corallina, enzyme induction in, 87

Nocardia opaca, nitrobenzoates and, 409

Nomadacris,
- ethereal sulfates in, 421
- glucosides in, 415

Nonadecapeptide, melanotropic activity of, 329

Noradrenaline, invertebrates and, 253–255

Nosopsyllus fasciatus, iron requirement of, 179

Nostoc, molybdenum and, 183

Nostocaceae, Venus and, 134

Notonecta,
- ethereal sulfates in, 421
- extraintestinal digestion in, 388
- glucosides in, 415

Notostracans, enzyme repression in, 99

Nucleic acid(s),
- hepatopancreas and, 382
- magnesium requirement and, 175
- metabolism, metamorphosis and, 232
- precursors, germination and, 5

Nucleosides, growth inhibition by, 166
Nutrition, comparative, *see* Comparative nutrition
Nutrition,
  extraterrestrial bodies and, 133–135
  free energy and, 141–148
  requirements, quantitation of, 125–126
  structural organization and, 148–169
Nymphalis antiopa, DDT metabolism in, 440

O

Ochromonas malhamensis, 202
  adenylcobamide and, 201
  juvenile hormone in, 281
Octadecapeptide, melanotropic activity of, 329
Octapeptide, melanotropic activity of, 329
Octopus,
  acetylcholine effect on, 250
  epistellary body of, 271
  extraintestinal digestion in, 389
Odonata,
  hemolymph, ions in, 140
Oleate,
  biotin and, 196
  requirement of, 193
Oligochaete,
  hemoglobin of, 100
  juvenile hormone in, 280
  neurosecretion in, 259–260
Omasum,
  fatty acids and, 363
  inner structure of, 361
Ommochromes,
  metamorphosis and, 233
  tryptophan and, 158, 228, 233
Omnivores, intestinal pH in, 360
Oncopeltus fasciatus, DDT metabolism in, 439–440
Oncorhynchus tschawytsha, uridine diphosphate glucuronic acid in, 448
Onobrychis sativa, rhodanese and, 434
Orchestia gamarella, androgenic gland, of, 271
Organic acids,
  insect blood and, 140, 141
  osmotic balance and, 138
  photosynthesis and, 142, 143
  plant sap and, 136
Organic molecules,
  complex, formation of, 132–133
  simple, origin of, 130–132
Organisin, properties of, 258
Organophosphates, detoxification of, 435
Ornithine,
  arginine and, 154
  conjugations, development and, 445–446
  detoxification and, 427–432
  enzyme repression and, 97
  glutamate and, 150
  nicotinamide synthesis and, 188
  pupa and, 228
  spermine synthesis and, 211
  synthesis, inhibition of, 77
Ornithine transcarbamylase, amphibian differentiation and, 61
Ornithodorus coriaceous, DDT metabolism in, 440
Ornithuric acid(s), formation of, 427–428
Oronectes immunis, juvenile hormone and, 280
Orotic acid,
  assay of, 165
  synthesis, repression of, 102
Orotidylate,
  product inhibition by, 78
  uridylate formation from, 78
Orotidylate decarboxylase, oroticaciduria and, 102
Orotidylate pyrophosphorylase, oroticaciduria and, 102
Orthoptera,
  amino acid requirement of, 160
  ecdysone action in, 275
  glucosides in, 415
  hemolymph, ions in, 140
Oscillatoria, copper requirement of, 180
Oscillatoriaceae, sulfur bacteria and, 144
Osmoregulation, development of, 135–136
Ostrea,
  acetylcholine effect on, 250
  heart, adrenaline and, 254
  iron uptake by, 355

Oviposition, neurosecretions and, 260, 261
Oxalacetate,
  aspartate formation from, 150
  enzyme repression by, 96
  spore respiration and, 15
  succinic dehydrogenase inhibition by, 80
Oxalate, thiamine and, 195
Oxalis,
  sap, pH of, 136
Oxamycin, 151
Oxidase(s),
  copper and, 180
  induction of, 83
Oxidation, detoxification and, 403–413
Oxidative dealkylation, detoxification and, 410–411
N-Oxides, detoxification and, 411
Oxybiotin, activity of, 196
Oxygen,
  enzyme induction and, 19, 90
  enzyme repression by, 99–100
  glycolysis and, 80
  intestinal absorption and, 349
  Mars and, 134
  primitive biosphere and, 129–130
  solar energy and, 142
  spore germination and, 5–6
  triosephosphate dehydrogenase and, 80
  Venus and, 134
Oxytocic activity,
  assay of, 302
  chick and, 303
  mammals and, 302–303
  species and, 314
Oxytocic hormone, subneural gland and, 283
Oxytocin,
  activities, species and, 305
  birds, 307
  chemical synthesis of, 310
  purification of, 304–307
  structure of, 309–310
Oyster,
  intestinal absorption in, 355
  neurosecretion in, 261
  shell formation in, 168

P

Palaemonetes,
  chromatotropin of, 264
  juvenile hormone and, 280
Palaemon serratus, chromatotropins and, 264
Palmitate, intestinal absorption of, 356
Palmityl aldehyde, sphingosine synthesis from, 182
Pancreas,
  bird, enzymes of, 371
  cephalopods and, 382
  frog, amylase in, 374
  secretion of, 364
  structure, fish and, 374–375
  zinc and, 178
Pandalus borealis, chromatotropin of, 264
Panspermia hypothesis, 128
Pantoate, biosynthesis of, 198
Pantothenate,
  biosynthesis of, 198
  function of, 198
  microorganisms and, 170
  occurrence of, 170
  requirement for, 198–199
Papain,
  arginine vasopressin and, 310
  chromatotropins and, 264
Papilio xuthus, pigmentation of, 234
Paracentrotus,
  eggs, calcium and, 40
Paracolobactrum aerogenoides,
  histidase, induction of, 86
Parakeratosis, zinc and, 179
Paraldehyde fuchsin, neurosecretory cells and, 267
Paramecium,
  acetylcholine in, 249
  metabolic rate of, 148
Paramecium aurelia,
  purine and pyrimidine requirement of, 166
  sterol requirement of, 208
Paramecium multimicronucleatum,
  purine and pyrimidine requirement of, 166
Parapepsins, significance of, 358

Parasites, carbohydrate requirement of, 146

Parasitism, castration by, 270

Parasympathetic system, gastric secretion and, 370

Parathelphusa,
  amylase, temperature and, 361

Parathion, oxidation of, 410

Parathormone, calcium deposition and, 169

Parenterals, gut of, 389

Pars intercerebralis, neurosecretory cells of, 266–267

Partition ratio, toxicity and, 413

Parvobacteriaceae, enzyme induction in, 84, 86

Pasteurella, nicotinamide and, 188

Pasteurella pestis,
  enzyme induction in, 86
  oxygen-induced enzymes of, 19
  porphyrin and, 190

Pasteurella tularensis, polyamine requirement of, 210–211

Pasteur effect,
  adaptive value of, 80
  distribution of, 80–81
  regulation by, 80

Peas,
  ω-aryloxyalkylcarboxylic acids and, 406
  glucuronyl transferase and, 420
  root, thiamine and, 165
  seed,
    germination of, 32
    triosephosphate dehydrogenase of, 80

Peanut, germination of, 32

Pecan tree, zinc deficiency in, 178

Pectates, magnesium and, 176

Pecten,
  heart, adrenaline and, 254

Pectin, synthesis of, 162

Pectinesterase, induction of, 89

Pediculus humanus, DDT metabolism in, 440, 441

Pelargonium hortorum, glycosides in, 416

Pelecypoda, juvenile hormone and, 280

Pellagra, nicotinamide and, 188

Pelmatohydra oligactis, juvenile hormone in, 280

Penicillin, metabolism of, 435–436

Penicillinase,
  induction of, 85, 86
  spores and, 17
  ultraviolet and, 19

Penicillium,
  calcium and, 175
  methylation in, 426

Pennaria tiarella, juvenile hormone in, 280

Pentachlorocyclohexane, formation of, 437

Pentachloronitrobenzene, detoxification of, 432

Pentachlorophenol,
  esters, fungicidal activity of, 435

Pentapeptide, melanotropic activity of, 329

Pentobarbital, oxidation of, 446

Pentosan, digestion of, 372

Pentose,
  formation, sea urchin eggs and, 48–50

Pepsin,
  collagen and, 388
  components of, 357–358
  corticotropin and, 325
  esophagus and, 373
  fibrin digestion by, 356
  fish and, 376
  invertebrates and, 382–383
  occurrence of, 390–391
  pH optimum of, 357–360
  proventriculus, 367

Peptidases,
  appendices pyloricae and, 376
  seed germination and, 32
  spore germination and, 9

Peptides,
  detoxification and, 427–432
  enzyme stability and, 17
  insect development and, 229–230
  spore germination and, 9–10

Peranema trichophorum, sterol requirement of, 209

Perch,
  appendices pyloricae in, 375, 376
  glucuronides and, 414

Perikaryons, acetylcholine and, 251–252
Periostracum, iodine and, 284
Periplaneta,
  acetylation in, 425
  glucose absorption in, 355
  phenols and, 415
Periplaneta americana,
  DDT metabolism in, 440, 441
  nicotine and, 412
  sex attractant of, 287
Periplanetin, monosaccharide in, 162
Peritrophic membrane, secretion of, 382
Permeability, slime mold differentiation and, 24–26, 65
Permease(s), induction of, 85, 88, 90
Pernicious anemia, pyrimidine synthesis in, 102
Perosis, manganese and, 182–183
Peroxidase(s),
  iron and, 179
  phenylacetaldehyde and, 405
  plant development and, 31
  porphyrins and, 189
Peroxide, photosynthesis and, 142
Petroleum, carbon balance and, 127
Petromyzon,
  digestive tract, spawning and, 377
pH,
  spore germination and, 5
  sporulation and, 12–13
Phaenicia cuprina, sodium requirement of, 174
Phaeophyta, enzyme induction in, 88
Phagocytosis,
  digestion and, 378–381
  food uptake and, 355
  particle size and, 378
Phalangiidae, molting hormone in, 262, 271
Pheasant, gizzard pH in, 370
  phenothiazine and, 410
Phenacetin, excretion of, 423
Phenol(s),
  conjugate, DDT metabolism and, 440
  detoxification of, 406, 409, 414, 416, 418, 419, 421, 422
  sulfate and, 420
Phenolase, detoxification and, 406
Phenol glucuronides, β-glucuronidase and, 419
Phenoloxidase,
  activation of, 235, 236
  copper and, 180
  epicuticle and, 234
Phenolphthalein, detoxification of, 414
Phenothiazine, detoxification of, 406–407, 409–410
Phenoxyacetic acid, oxidation of, 409
Phenylacetaldehyde, oxidation of, 405
Phenylacetic acid,
  detoxification of, 427–431
  glutamine and, 427
Phenylalanine,
  arginine vasopressin and, 310–311
  insulin and, 333
  oxidation, ascorbate and, 194
  pupa and, 228
  requirement for, 156, 159–163
  spore germination and, 7
  synthesis of, 156
  transport of, 351
Phenylalanine^3-arginine^8-oxytocin, *see* Arginine-vasopressin
Phenylalanine hydroxylase, repression of, 103
Phenylalanine^3-lysine^8-oxytocin, *see* Lysine-vasopressin
Phenylhydrazine, acetylation of, 425
Phenylketonuria, etiology of, 157
γ-Phenylpropionic acid, detoxification of, 428
Phenylpyruvate, utilization of, 156
Phenylthiourea, toxicity of, 410
Pheochrome cells, annelids and, 254
Pheromones,
  action of, 248
  definition of, 285–286
Phlebobranchiata, vanadium in, 185
Phlorin, formation of, 418
Phlorizin,
  amino acid absorption and, 351
  monosaccharide absorption and, 351
  water absorption and, 352
Phloroglucinol, detoxification of, 416, 418
Pholcus, juvenile hormone in, 280
Phormia, nicotinamide and, 187
Phormia regina,
  amino acid requirement of, 161
  choline and, 204

  methionine and, 153
  sterol requirement of, 209
Phormidium tenue, copper requirement of, 180
Phosphagens,
  arginine and, 154–155
  spore germination and, 9
Phosphatase,
  acid, amphibian differentiation and, 58, 61
  alkaline,
    amphibian differentiation and, 58, 61
    differentiation and, 57
    induction of, 92
    repression of, 96–98, 103
    vitamin D and, 209
    zinc and, 178
  detoxification and, 435, 443
  product inhibition of, 79
  repression of, 98–99
  serum, sterols and, 209
Phosphate,
  absorption, vitamin D and, 209
  energy-supplying pathways and, 79
  enzyme repression by, 96
  insect blood and, 140
  molybdenum and, 183
  morphogenesis and, 27
  osmotic balance and, 138, 139
  Pasteur effect and, 80
  plant sap and, 136
  product inhibition by, 79
  sea urchin egg development and, 42
  surface water, origin of, 130
  uptake of, 355
Phosphatidylserine, formation of, 151
3′-Phosphoadenosine diphosphate, coenzyme A and, 198
3-Phosphoadenosine-5-phosphosulfate, ethereal sulfates and, 422
Phosphoarginine, 154
Phosphocreatine, arginine and, 154
Phosphoglucoisomerase, inhibition of, 81
6-Phosphogluconate, phosphoglucoisomerase and, 81
Phosphogluconic dehydrogenase, manganese and, 181–182
  sea urchin eggs and, 48–50, 65
3-Phosphoglyceraldehyde, photosynthesis and, 142
3-Phosphoglycerate,
  glycine and, 149
  serine formation from, 150–151
Phosphohexokinase, spores and, 15
Phospholipids,
  choline and, 204
  inositol and, 210
  spore germination and, 9
Phosphoprotein,
  egg, autolysis of, 55
Phosphoprotein phosphatase, amphibian differentiation and, 55–56, 65
Phosphoramides, oxidation of, 411
5-Phosphoribosyl-1-pyrophosphate, tryptophan synthesis and, 157
Phosphorothioates, oxidation of, 410, 411
Phosphorus,
  balance of, 127
  calcium deposition and, 169
  metabolism, metamorphosis and, 232
Phosphorylase,
  activation of, 105
  inactive form, 104
Phosphorylation,
  monosaccharide absorption and, 350
  oxidative,
    magnesium and, 176
    vitamin K and, 191
Phosphoserine phosphatase, product inhibition of, 79
Photoreceptivity, vitamin A and, 205, 206
Photosensitization, phenothiazine and, 409–410
Photosynthesis,
  carbon balance and, 126–127
  glucose produced by, 126
  lipoate and, 199
  oxygen and, 130
  vitamin B_{12} and, 203
Photosynthetic autotrophs,
  amino acid requirements of, 158
  free energy of, 141–143
Phototropism, riboflavin and, 189
Phthiocol, occurrence of, 191
Phycocyanin, sodium and, 174
Phycomyces,
  carotene synthesis in, 152

nicotinamide and, 188
Phycomycetes, lysine biosynthesis by, 124
Phyllodecta, salicin and, 405
Physalia,
  5-hydroxytryptamine in, 255
  juvenile hormone in, 280
Physcosoma japonicum, neurosecretion in, 260
Physcosoma lanzarotae, neurosecretion in, 260
Phytoflagellates,
  amino acid requirements of, 158
  mineral deposits in, 167
  pyrimidine requirement of, 166
Picolines, detoxification of, 404
Picolinic acid, detoxification of, 429, 430
Picolinuric acid, formation of, 430
Pig, *see also* Hog
  amino acid requirement of, 163
  aromatic amino acid hydroxylation by, 445
  digestion in, 366
  ethereal sulfates in, 420
  hippuric acid in, 427
  phenothiazine and, 409
  zinc deficiency in, 179
Pigeon,
  acetylation in, 424
  benzoic acid and, 428, 429, 432
  conditioned reflexes in, 371
  demethylation by, 411
  gastric secretion,
    pepsin in, 371
    stimulation of, 370–371
  gizzard pH in, 370
  liver,
    feedback inhibition in, 78
    product inhibition in, 79
Pigment(s),
  biosynthesis, metamorphosis and, 233–234
  formation,
    iron and, 179
    magnesium and, 176
    salt and, 138
  melanin-like, lysine and, 155
Pike, gastric pH in, 359
Pilocarpin, pancreatic secretion and, 364
Pilobolus, coprogen and, 190
Pilocarpine,
  pancreas and, 374
  pepsin secretion and, 373
Pimelic acid, biotin and, 196
Pinguicula, proteolysis by, 390
Pinnaglobin, manganese and, 182
Pinna squamosa, pigment of, 182
Pinocytosis, particle size and, 378
Pinus resinosa, glycosides in, 416
Pinus sativa, glycosides in, 416
Piperonyl butoxide, pyrethrin synergism and, 435, 441
Pityrosporum ovale, oleate and, 193
Placenta, acetylcholine and, 249
Plagiodera, salicin and, 405
Plaice, fat absorption by, 377
Planaria,
  acetylcholine and, 249, 253
  phagocytosis in, 380
Planorbis, enzyme repression in, 99
Plants,
  acetylation in, 425
  acetylcholine in, 249
  acetyl indoxyl and, 412
  aldrin and, 411
  amino acid requirements of, 158, 159
  aromatic acids and, 430
  aromatic hydroxylation in, 406
  ω-arylalkylcarboxylic acids and, 405–406
  biotin and, 196–197
  calcium deficiency in, 175
  carnivorous, digestion in, 390
  chloride requirement of, 177
  citrulline and, 154
  comparative nutrition of, 122
  copper deficiency in, 180
  detoxification in, 404
  enzyme repression in, 98, 99
  fluid medium maintenance in, 136–138
  glycosides in, 413, 416–418
  heptachlor and, 411
  heterotrophism in, 171–172
  inactive enzyme in, 105
  iodine requirement of, 177
  iron deficiency in, 179
  juvenile hormone in, 281
  lysine biosynthesis by, 124
  magnesium deficiency in, 176

manganese deficiency in, 182
methylation in, 426
molybdenum deficiency in, 183
nicotinamide and, 187, 188
osmotically active materials in, 212
pantothenate and, 198
Pasteur effect in, 81
phenylacetaldehyde and, 405
phosphoramides and, 411
phosphorothioates and, 410
photosynthesis, oxygen and, 142
polyunsaturated acids and, 193
potassium deficiency in, 174
purine and pyrimidine synthesis by, 164–166
rhodanese in, 434
sterols of, 208
Systox and, 410
thiamine and, 195
tissues, oxygen consumption of, 148
tryptophan synthesis in, 157
vitamin A in, 206, 207
vitamin B_6 and, 197
vitamin B_{12} and, 202
zinc deficiency in, 178

Plasmin, activation of, 105
Plasmodium knowlesii, purine and pyrimidine requirement of, 166
Platanus orientalis, glycosides in, 416
Platelets, 5-hydroxytryptamine in, 255
Platyhelminthes,
  juvenile hormone and, 280
  phagocytosis in, 380
Platysamia cecropia, see *Hyalophora cecropia*
Plodia interpunctata, DDT metabolism in, 440
Pneumococci,
  capsules, polysaccharide synthesis and, 162
Polarized light, asymmetric syntheses and, 131
Pollachius virens, neurohypophyseal hormones of, 308
Pollack,
  pituitary powder, activity of, 314
Polycelis nigra, ocular regeneration in, 257–258
Polychaete,
  hemoglobin of, 100
  juvenile hormone in, 280
  neurosecretion in, 258–259
Polygalacturonase, induction of, 89
Polyglycine, formation of, 132
Poly-β-hydroxybutyrate, sporulation and, 13, 18
Polymerization, prebiotic conditions and, 132–133
Polymixin B, nitrate reductase formation and, 91
Polynucleotides,
  synthesis,
    insects and, 167
    plants and, 164–166
    protozoa and, 166
    vertebrates and, 167
Polypeptidase(s), cephalopod, 382
Polyphenoloxidase, plant development and, 31
Polyphosphate(s), spore germination and, 9
Polyplacophora, neurosecretion in, 260
Polysaccharides,
  components of, 162
  synthesis, requirements for, 161–162
Polytoma uvella,
  enzyme induction in, 89
  hydroxyapatite in, 168
Polytomella caeca, hydroxyapatite in, 168
Popillia japonica,
  amino acid nitrogen in, 228
  metamorphosis, respiration and, 224
Porifera,
  acetylcholine and, 249
  juvenile hormone and, 280
  neurosecretion in, 257
Porphobilinogen, formation of, 150
Porphyrin(s),
  electron transport and, 189–190
  glycine and, 150
  synthesis, repression of, 96
  vanadium complex, occurrence of, 186
Porthetria dispar, sex attractant of, 286–287
Potassium,
  insect blood and, 140
  ion antagonism and, 186
  membrane permeability and, 352
  osmotic balance and, 138, 139
  plant sap and, 136

requirement for, 174–175
rumen and, 363
sea urchin egg development and, 45
sodium requirement and, 174
surface water, origin of, 130
rubidium and, 186
Potato, ascorbate and, 194
Pout,
pituitary powder, activity of, 314
Pregnancy, iron requirement and, 180
Primates,
ascorbate and, 194
comparative nutrition of, 122
saliva, amylase in, 357
Proconvertin, vitamin K and, 191
Procuticle, composition of, 234
Prodigiosin, iron and, 179
Product inhibition, examples of, 78–79
Proline,
arginine vasopressin and, 310–311
arginine-vasotocin and, 312
biosynthesis of, 153
enzyme repression by, 98
glutamate and, 150
insect blood and, 140
β-melanocyte-stimulating hormone and, 323, 324
oxidation, ascorbate and, 194
oxytocin and, 309
pupa and, 227, 228, 229
requirement for, 159–161
sclerotization and, 234
transport of, 351
Propionate,
abiogenic formation of, 131
ruminant and, 362
Propionibacterium shermanii, cobamide of, 201
Propionic acid bacteria, Pasteur effect in, 81
Propylarsonic acid, methylation of, 426
n-Propylbromide, detoxification of, 433
Protamines, arginine and, 155
Protease(s), *see also* Proteinase(s)
enzyme lability and, 65
gonadotropic hormone and, 282
loss of, 389
molting and, 230
sea urchin eggs and, 40
starvation and, 14
Protein(s),
amphibian differentiation and, 53–55
crystalline style and, 384
ecdysone activity and, 272–273
egg formation and, 230
enzyme inhibitors and, 105
metabolism, metamorphosis and, 227–231
nutritional requirement for, 149–161
osmotic balance and, 138
pattern, amphibian differentiation and, 56–57
peritrophic membrane and, 382
sclerotization and, 234
sea urchin egg development and, 41
seed germination and, 35–36
skeletal, glycine and, 149
structural, hydroxyproline and, 153
synthesis,
ecdysis and, 226
ecdysone and, 238–239
enzyme induction and, 84–87, 94
insulin and, 332
permease induction and, 88
rate of, 106
sea urchin development and, 42–45
slime mold differentiation and, 26
utilization,
energy and, 147
pseudoplasmodium and, 21–23
water metabolism and, 139
Proteinase(s), *see also* Protease(s)
bile acids and, 360
cephalopod, 382
enzyme activation by, 105
inactive forms of, 104
salivary, 382
secretion, protein and, 385
seed germination and, 32
sperm and, 40, 65
Proteinoids, abiotic formation of, 133
Proteolysis,
inhibition of, 64
spore germination and, 9
sporulation and, 14
starvation and, 14
Proteus,
hydrogenase, activation of, 104
juvenile hormone in, 281

Prothoracic gland,
  brain hormone and, 266
  hormone of, 222, 265, 272
Prothoracotropic hormone, *see* Brain hormone
Prothrombin, vitamin K and, 191
Protists, *see also* Microorganisms
  alanine requirement of, 150
  amino acid requirements of, 158
  aspartate and glutamate requirement of, 150
  serine requirement of, 151
Protocatechuic acid,
  detoxification of, 415
  o-diphenoloxidase and, 236
  formation of, 409
Protocatechuic oxidase, induction of, 89
Protocerebron, neurosecretory cells of, 266–267
Protochordata,
  nonneural hormones in, 282–285
  relationships of, 282
Protogen, *see* Lipoic acid
Protoplasts, spermine and, 211
Protoporphyrin IX, requirement for, 190
Protozoa, *see also* Microorganisms, Protists
  acetylcholine and, 248
  amino acid requirements of, 160
  calcium requirement of, 175
  cellulase in, 387
  citrulline and, 154
  comparative nutrition of, 122
  digestion in, 378, 379
  ecdysone and, 277
  enzyme induction in, 89
  enzyme repression in, 98
  glycine requirement of, 149
  marsupials and, 367
  mineral deposits in, 167–168
  purine and pyrimidine requirements of, 166
  ruminants and, 362, 364
  sterols and, 208–209
Proventricle, neurosecretion and, 259
Proventriculus,
  function of, 368–370
  pH of, 370
  secretions of, 367
Provitamin(s) A,
  conversion of, 207
  occurrence of, 206
Pseudocobalamins, adenine and, 164
Pseudoemys elegans, acetylation in, 424
Pseudomonadaceae, enzyme induction in, 84, 86
Pseudomonad(s),
  carbon monoxide utilization by, 145
  lysine biosynthesis by, 123–124
Pseudomonas,
  calcium and, 175
  enzyme induction in, 84
  enzyme repression in, 96
  naphthalene and, 408
  permeases, induction of, 85
Pseudomonas aeruginosa, iron requirement of, 179
Pseudomonas beijerinckii, salt and, 138
Pseudomonas fluorescens,
  inducible enzymes, number of, 106
Pseudomonas ovalis,
  carboxydismutase, induction of, 86
Pseudoplasmodium,
  differentiation of, 45
  formation of, 20
  respiration of, 21–22
Pseudosarcophaga affinis,
  amino acid requirement of, 161
  potassium and, 175
  vitamin B_6 and, 198
Pteridines,
  brain hormone and, 268
  folic acid synthesis and, 200
Pterins, thiamine and, 195
Pterostichus, glucosides in, 415
Pteroylglutamic acid, *see* Folic acid
Punicin, occurrence of, 177
Pupa, transformations in, 222
Puparia,
  formation, cystine and, 152
Pupation, free amino acids and, 227–228
Purine(s),
  analogs, 166
  biosynthesis, aspartate and, 150
  folic acid and, 199, 200
  histidine and, 164
  microbiological assay of, 165
  pteridines and, 200
  requirement for, 164

synthesis,
    antibiotics and, 151
    repression of, 96, 102
  threonine requirement and, 151
Purine nucleotides, feedback inhibition by, 78
Putrescine, polyamine requirement and, 210–211
Pyrausta nubitalis, DDT metabolism in, 440
Pyrethrins,
  inactivation of, 434–435
  toxicity, temperature and, 441
Pyridine, methylation of, 425
Pyridine nucleotides, nitroreductase and, 413
Pyridoxal, *see also* Vitamin B_6
  amino acid uptake and, 351
  nonenzymic catalysis by, 171
  microorganisms and, 170
Pyridoxal kinase, distribution of, 197
Pyridoxal phosphate, glycine formation and, 149
Pyrimidine(s),
  analogs, 166
  enzyme repression by, 96, 98, 102
  folic acid and, 199
  microbiological assay of, 165
  requirement for, 164
  synthesis, repression of, 95–96
  thiamine and, 164
Pyrimidine deoxyribonucleotides, feedback inhibition and, 78
Pyrochroa, glucosides in, 415
Pyrogallol, detoxification of, 416
Pyrroline-5-carboxylate reductase, repression of, 98
Pyrrophyta, enzyme induction in, 88
Pyruvate,
  acetoin formation from, 171
  alanine formation from, 150
  isoleucine synthesis and, 152
  lipoate and, 199
  spore germination and, 5, 7, 8
  sporulation and, 13
  thiamine and, 195
  valine synthesis and, 152
Pyruvate kinase, potassium and, 174, 175

Q

Queen substance, chemical nature of, 288
Quinaldine, detoxification of, 404
Quinine oxidase, occurrence of, 412, 446–447
Quinol, detoxification of, 416, 419
Quinoline(s), methylation of, 425
Quinolinic acid, nicotinamide synthesis and, 187
8-Quinolinol, detoxification of, 421
Quinones,
  electron transport and, 190–192
  sclerotization and, 234–235

R

Rabbit(s),
  acetamido compounds and, 423
  acetylation in, 423
  p-aminosalicylic acid and, 427
  anthracene and, 407
  aromatic acids and, 442
  aromatic amino acid hydroxylation by, 445
  benzene hexachloride and, 437–438
  chitinase and, 361
  chlorobenzene and, 407
  crude fiber digestion by, 367
  DDT metabolism in, 438
  dehalogenation in, 436
  digestion in, 366
  ear, acetylcholine analogs and, 253
  enzyme induction in, 92, 94
  ethereal sulfates in, 422
  fibroblasts, amino acid requirement of, 162
  glucuronides in, 414
  hippuric acid in, 427
  insulin assay in, 331
  insulin of, 336
  mercapturic acids in, 432–433
  methoxychlor and, 438
  methylation in, 426
  milk-ejecting activity and, 303, 305
  naphthalene and, 407
  β-naphthylamine and, 407, 420
  nicotine and, 412
  nitroreductase in, 446
  phenols and, 409

  quinine oxidase in, 412
  rhodanese in, 434
  tissues, serine and, 151
Radiolaria, skeletons of, 167–168
Radium,
  accumulation of, 187
  cell growth and, 186
Raffinose, seed germination and, 32–33
Ragweed, selenium in, 186
Rana, ethereal sulfates in, 421
Rana catesbeiana,
  acetylation in, 424
  serum, electrophoresis of, 56
Rana clamitans,
  carbamyl phosphate synthetase, isolation of, 62–63
Rana esculenta,
  neurohypophyseal hormones of, 307–308
  sugar absorption by, 354
Rana pipiens,
  acetylation in, 424
  enzyme induction in, 92
  glucuronides in, 414
Raphanus sativus, glycosides in, 417
Rat(s)
  2-acetamidofluorene and, 407
  acetylation in, 423
  adrenal, hog corticotropin and, 316
  amino acid requirement of, 162
  amino acid transport in, 351
  p-aminosalicylic acid and, 427
  anthracene and, 407–408
  antidiuretic activity and, 303
  arginine and, 154
  aromatic amino acid hydroxylation by, 445
  bromide and, 177
  chlorobenzene and, 407
  DDT metabolism in, 438
  dechlorination in, 437
  energy requirement of, 147
  enzyme induction in, 92, 94
  enzyme repression in, 100–101, 103
  epididymus, insulin and, 331–332
  fetal,
    ethereal sulfates in, 444
    rhodanese in, 444
  fluoride and, 176
  gastric pH in, 359
  glucuronides in, 414
  glutamine acylation in, 431
  heptachlor and, 411
  hippuric acid in, 427
  hydrocarbon oxidation by, 146
  iodine requirement of, 178
  juvenile hormone in, 281
  liver, product inhibition in, 78, 79
  mercapturic acids in, 432–433
  molybdenum deficiency in, 184
  naphthalene and, 407–408
  β-naphthylamine and, 407
  naphthylthiourea and, 410
  posterior pituitary powder, activity of, 314
  quinine oxidase in, 412
  rhodanese in, 434
  selenium and, 427
  sugar absorption in, 354
  tissues,
    amino acid requirements of, 162
    oxygen consumption of, 148
  uterus, oxytocic activity and, 302, 303, 305
  vasopressin of, 307
  vasopressor activity and, 303, 305
  xanthopterin and, 200
  xanthurenic acid and, 427
  zinc requirement of, 179
Ray,
  pituitary powder, activity of, 314
Reaction rates, detoxification and, 441–443
Receptors,
  acetylcholine and, 252, 253
  adrenaline and, 255
Renal tubule, arginine-vasotocin and, 316
Rennin, pH optimum of, 358, 359
Reptiles,
  acetylation in, 424–425
  aromatic acids and, 428–431
  corticotropin and, 318
  digestion in, 372–373
  ethereal sulfates in, 420, 421
  glucuronides in, 414
  insulin action in, 332

- neurohypophyseal hormone activity in, 303
- nitro groups and, 413
- tapetum lucidum of, 164
- uricotelism in, 432
- vitamin A of, 206

Resorcinol, detoxification of, 416

Respiration,
- amphibian differentiation and, 52–55, 58–59
- detoxification and, 419
- egg homogenates and, 57
- enzymes, induction of, 89
- germination and, 5–8, 10
- gonadotropic hormone and, 282
- injury and, 226
- metamorphosis and, 223–227
- sea urchin egg development and, 38–41
- seed germination and, 30–31, 36–37
- slime mold differentiation and, 21–22
- sporulation and, 12–13

Respiratory adaptation, sporulation and, 19

Respiratory quotient,
- amphibian differentiation and, 53–54
- seed germination and, 32, 35

Reticulum, inner surface of, 361

Retina, oxygen consumption of, 148

Retinine reductase, vitamin A and, 207

Rhamnose, glycoside formation and, 418

Rhinolophus ferrum equinum, chitinase in, 361

Rhizobium,
- calcium and, 175
- chromium and, 186
- molybdenum and, 183

Rhizopterin, activity of, 200

Rhizopus nigricans, pterin from, 200

Rhodanese,
- cyanide and, 433–434
- development and, 444
- occurrence of, 434

Rhodnius,
- ecdysone assay and, 272
- gonadotropic hormone in, 279
- juvenile hormone and, 277
- molting of, 230

Rhodnius prolixus,
- ecdysone action in, 275, 276
- molting cycle, metabolism and, 226

Rhodommatin, synthesis of, 233

Rhodophyta, enzyme induction in, 88

Rhodopseudomonas spheroides,
- catalase induction in, 85, 86
- enzyme repression in, 96

Rhodotorula rubra, thiamine and, 195–196

Rhyncocoela, juvenile hormone in, 280

Riboflavin,
- biosynthesis of, 189
- electron transport and, 189
- nonenzymic reactions of, 171
- pteridines and, 200
- requirement for, 189, 203
- tapetum lucidum and, 164

Ribonucleic acid,
- arginase and, 51
- amphibian differentiation and, 58
- chromosome puffing and, 237
- enzyme inhibition by, 105
- enzyme stability and, 17
- metamorphosis and, 232
- requirement for, 166, 167
- sea urchin egg development and, 42
- synthesis, sporulation and, 18–19

Ribose-5-phosphate, histidine synthesis and, 155

Ribosidase, spore germination and, 8

Ribulose, vitamin B_{12} and, 203

Ribulose-1,5-diphosphate, photosynthesis and, 142

Rickettsia, *p*-aminobenzoate and, 201

Roaches, *see also* Cockroach
- endosymbionts in, 389–390

Rodentia,
- saliva, amylase in, 357

Roots, thiamine pyrimidine and, 164–165

Rootlets, oxygen consumption of, 148

Root tips, oxygen consumption of, 148

Rosette leaf disease, zinc and, 178

Royal jelly, queen production and, 288

Rubidium,
- effects of, 186
- potassium requirement and, 174

Rumen,
- volatile fatty acids and, 362, 363
- volume of, 361

Ruminant,
- bacteria, food quality and, 363–364

  cobalt and, 184
  crude fiber digestion by, 367
  digestion in, 361–366
  methylation in, 426
  wild, digestion in, 364

S

Saccharase, induction of, 89
Saccharomyces,
  enzyme induction in, 89–90
  enzyme repression in, 98–99
  potassium and, 174
  product inhibition in, 78
  tryptophan synthesis in, 157
Saccharomyces cerevisiae,
  feedback inhibition in, 77
  ubiquinone of, 190
Saccharomyces drosophilarum, enzyme repression in, 99
Saccoglossus kowalevskii, juvenile hormone in, 281
Salamandra,
  ethereal sulfates in, 421
  glucuronides in, 414
Salamander, demethylation in, 411
Salicin, detoxification of, 405
Salicinase, fish and, 376
Salicyl alcohol, oxidation of, 405
Salicylaldehyde,
  detoxification of, 416
  secretion of, 405
Salicylic acid,
  detoxification of, 429
  formation from naphthalene, 408
Saligenin, detoxification of, 416
Saliva,
  amylase in, 357
  mucin in, 357
Salivary gland(s),
  acid secretion by, 383
  chromosomes, puffing of, 237
  5-hydroxytryptamine in, 255, 256
  invertebrates and, 382
Salix purpurea, glycosides in, 417
Salmon,
  digestive tract, spawning and, 377
  pepsin of, 376
  vitamin A of, 206
Salmonella,
  endotoxin, monosaccharides of, 162
  histidine synthesis by, 156
  tryptophan synthesis in, 157
Salmonella typhimurium, feedback inhibition in, 77
Salmonella typhosa, amino acid requirement of, 159
Saltsick, deficiencies in, 181
Salvelinus,
  gut, bacteria in, 376
Sambucus sieboldiana, glycosides in, 416
Samia walkeri, ecdysone action in, 275
Sap,
  osmotically active constituents of, 136–137
  pH of, 136
Saprolegniales, lysine biosynthesis by, 124
Sarcodina, mineral deposits in, 167
Sarcophaga bullata, juvenile hormone in, 280
Sarcophaga crassipalpis, DDT metabolism in, 440
Sarcosine,
  abiogenic formation of, 131
  glycine and, 150
Sarracenia, prey capture by, 390
Scale(s), riboflavin in, 189
Scaphiopus holbrookii, acetylation in, 424
Scenedesmus,
  hydrogenase, chloramphenicol and, 88
  molybdenum and, 183
Schistocerca,
  ethereal sulfates in, 421
  glucose absorption by, 355
  glucosides in, 415
Schistocerca gregaria,
  ascorbate and, 194
  vitamin A and, 207
Scinus officinalis, aromatic acids and, 428
Sciobius granosus, nucleic acid and, 167
Scleroproteins, iodine and, 284
Sclerotization,
  amino acids and, 229
  metamorphosis and, 234–237
Scopulariopsis brevicaulis, methylation in, 426
Scorpaena porcus, sugar absorption by, 354

Scorpion(s), 5-hydroxytryptamine in, 255
Scorpionida, hepatopancreas in, 381
Sculpin, acetylation in, 424
Scutellaria, glucuronides in, 413
Sea anemone,
  endosymbionts in, 389–390
  5-hydroxytryptamine in, 255
Sea urchin,
  egg development,
    altered enzymes and, 47–52
    double gradient theory, 45–47
    energy requirement for, 38–45
  eggs, ethereal sulfates and, 445
Sea water,
  bromine in, 177
  copper in, 180
  fluoride in, 176
  iodine in, 177
  pH of, 136
Secale cercale, rhodanese in, 434
Secretin,
  bird and, 371
  pancreatic secretion and, 364
Sedoheptulose, glycoside formation and, 418
Seeds,
  ergothionine in, 156
  germination,
    altered enzymes and, 36–38
    energy requirement of, 30–36
Selenium,
  compounds, methylation of, 426–427
  requirement for, 185
  tocopherol deficiency and, 192
  toxicity of, 185
Semen, ergothionine in, 156
Sepia officinalis, 5-hydroxytryptamine in, 255
Serine,
  biosynthesis of, 150
  cocoon spinning and, 227–228
  corticotropin and, 321
  cysteine synthesis and, 152
  detoxification and, 427
  glycine formation from, 149
  insulin and, 336
  lipid synthesis and, 163
  α-melanocyte-stimulating hormone and, 321
  β-melanocyte-stimulating hormone and, 319, 323, 324, 327
  product inhibition by, 79
  requirement for, 150–151, 159–162
  transport of, 351, 356
  tryptophan synthesis and, 157
D-Serine,
  occurrence, animals and, 151
Serine hydroxymethylase,
  glycine formation and, 149
  manganese and, 182
Serine-isoleucine-oxytocin, fish, 308
Serotonin, *see also* Hydroxytryptamine
  occurrence of, 157–158
Serratamic acid, serine and, 151
Serratia, serratamic acid in, 151
Serratia marcescens, iron requirement of, 179
Setonyx,
  digestion in, 366–367
  stomach, anatomy of, 366
Sex attractants, nature of, 286–287
Sex hormones, mollusks and, 270–271
Sexualization, neurosecretions and, 259
Sheep,
  abomasum, acid secretion by, 364
  acetylation in, 423
  ammonia utilization by, 364–365
  brain, product inhibition in, 78
  corticotropin of, 320–321, 325–326
  fetal, hippuric acid in, 445
  glucuronides in, 414
  hippuric acid in, 427
  insulin of, 336
  neurohypophyseal hormones of, 306, 307
  oxytocin of, 310
  pancreas, secretion of, 364
  phenothiazine and, 409–410
  plasma, chloride and, 363
  posterior pituitary powder, activity of, 314
  rhodanese in, 434
  vasopressin of, 311
Shikimic acid,
  p-aminobenzoate and, 201
  phenylalanine synthesis and, 156
  tryptophan synthesis and, 157
Shrew, energy requirement of, 147
Sialis lutaria, ecdysone action in, 275

Siderocapsa, free energy of, 145
Silicates, plant rigidity and, 167
Silicoflagellata, skeletons of, 167
Silicon,
  function of, 171
  requirement for, 186
Silk,
  digestion of, 388
  glycine and, 149
Silkworm,
  glandectomized, amino acids in, 227–228
  hemolymph, citrate in, 141
  3-hydroxyanthranilic acid and, 430
  nicotinamide and, 187
  vitamin A in, 207
Single-carbon,
  transfer, vitamins and, 199–205
Sinus gland, neurosecretion and, 261, 263
Sipunculids, acetylcholine in, 249
Sipunculoidea, neurosecretion in, 260
Sitosterol, requirement for, 208–209
Skate,
  gastric juice, secretion of, 374
Skin,
  hydroosmotic activity and, 303
  natriferic activity and, 304, 316
  oxygen consumption of, 148
Slime molds,
  carbohydrate requirement of, 163
  sporulation, 13, 14, 19–20
    altered enzyme activities and, 27–30
    energy requirement of, 20–27
    initiation of, 20
Sloth, stomach of, 367
Smerinthus,
  diapausing, amino acids in, 228
  ethereal sulfates in, 421
  glucosides in, 415
Snail,
  heartbeat, cations and, 139
  hemoglobin, oxygen and, 99, 100
Snake,
  aromatic acids and, 430
  corticotropin and, 318
  gastric pH in, 359
Sodium,
  chloride requirement and, 177
  insect blood and, 140
  ion antagonism and, 186
  membrane permeability and, 352
  osmotic balance and, 138, 139
  plants and, 137
  requirement for, 173–174
  rumen and, 363
  surface water, origin of, 130
Sodium chloride,
  amylases and, 383
  intestinal transport of, 353, 354
Soil,
  aldrin and, 411
  heptachlor and, 411
Solanaceae, glycosides in, 418
Solanum tuberosum, glycosides in, 417
Solenogastra, neurosecretion in, 260
Sorbose, intestinal absorption of, 349–350
Sorocarp, respiration of, 21, 22
Soybean, juvenile hormone and, 281
Sparrow, chitinase in, 361
Spasmodic activity, fish and, 304
Sperm,
  arginine and, 155
  proteolytic enzymes in, 40, 65
Spermatocytes, ecdysone assay and, 272–273
Spermatophyta, enzyme induction in, 90
Spermidine, requirement for, 210
Spermine,
  arginine and, 155
  glutathione and, 195
  requirement for, 210–211
  synthesis of, 211
Spermoviduct,
  glands, hormone and, 271
Sperm whale, insulin of, 336
Sphaerotilus, free energy of, 145
Sphingomyelin, synthesis of, 164
Sphingosine,
  lipid synthesis and, 164
  synthesis, manganese and, 182
Sphinx,
  development, amino acids and, 228
  ethereal sulfates in, 421
  glucosides in, 415
Spider(s),
  p-aminobenzoate and, 420
  ethereal sulfates in, 421
  extraintestinal digestion in, 389
Spinal cord, cholinesterase in, 59

Spirochetes, crystalline style and, 384
Spirogyra, ion antagonism in, 186
Spirostomum ambiguum, hydroxyapatite in, 168
Spirulina, copper requirement of, 180
Spisula,
  eggs, pyridine nucleotides in, 47–48
Sponges,
  acetylcholine and, 248, 249
  endosymbionts in, 389–390
  iodine fixation by, 284
  phagocytosis in, 380
  skeletons of, 168, 186
Spongins,
  amino acids in, 160
  iodine and, 284
Spores,
  germination,
    electron transport and, 10–11
    energy requirement of, 5–10
    initiation of, 5
Sporulation,
  altered enzyme activity and, 14–19
  calcium and, 175
  initiation of, 11–12
  potassium and, 174
  respiration and, 12–13
Squalene,
  leucine and, 152
  steroids and, 208
Squalus acanthias, acetylation in, 424
Squid, rhodanese in, 434
Squilla, skeleton of, 168
Stalk cells, spore formation and, 66
Stalk glands, function of, 271
Staphylococci, penicillin and, 436
Staphylococcus aureus,
  alkaline phosphatase of, 96
  β-galactosidase, induction of, 86
  uracil requirement of, 165
Starch,
  amylases and, 361
  amylase secretion and, 385
  seed germination and, 33
  stomach action on, 360
  synthesis of, 162
  urea utilization and, 362–363, 365
Starvation,
  glucuronide formation and, 414
  pseudoplasmodium formation and, 20
Stegobium paniceum, vitamin B_6 and, 198
Stemphylium sarcinaeformae, dimethoxynaphthalene and, 435
Stereokinase, fructose absorption and, 354
Sterility, tocopherol and, 192
Steroids,
  action mechanism of, 290
  leucine and, 152
  oxidation, ascorbate and, 194
  taxonomy and, 208
  vitamin, 208–210
Steroid dehydrogenase, induction of, 92
Sterol(s), sulfates of, 420, 422
Stigmasterol, requirement for, 208–209
Stolonization, neurosecretions and, 259
Stomach,
  secretion, stimulation of, 374
Strepogenin, nature of, 211
Streptococcus,
  hemolytic,
    adenine and, 165
    strepogenin and, 211
Streptococcus cremoris, lipoate and, 199
Streptococcus faecalis,
  folic acid replacement in, 166
  folic acid requirement of, 199
  lipoate and, 199
  malic enzyme, induction of, 87
  vitamin B_6 and, 197
Streptococcus lactis,
  lactate production, adenine and, 165
  rhizopterin and, 200
Streptomyces, O-carbamylserine in, 151
Streptomyces antibioticus, actinomycin I and, 154
Streptomyces aureofaciens, chlorine and, 177
Streptomyces griseus,
  threonine decarboxylation by, 152
  vitamin B_{12} and, 202
Streptomyces venezuelae, chlorine and, 177
Streptomycetaceae, enzyme induction in, 84
Strontium,
  accumulation of, 187
  calcium requirement and, 175
  ion antagonism and, 186

Strontium sulfate, skeletons and, 168
Structural organization,
  nutritional requirements,
    lipid, 163–164
    mineral deposits, 167–169
    polynucleotides, 164–167
    polysaccharides, 161–163
    proteins, 149–163
Subesophageal ganglion, hormone of, 269
Subneural gland, function of, 283
Substrate,
  availability, differentiation and, 30, 50, 65
  enzyme activation by, 103–104
Subtilisin, oxytocin and, 309
Succinate,
  abiogenic formation of, 131
  enzyme repression by, 96, 99
  germinating seeds and, 37
  insect blood and, 141
  isocitratase and, 81
  porphyrins and, 150
Succinic-cytochrome c-reductase, induction of, 90
Succinic dehydrogenase,
  amphibian differentiation and, 58, 59
  carbon dioxide and, 80
  metamorphosis and, 226
  oxalacetate inhibition of, 80
Succinoxidase,
  brain, cholinesterase and, 59–60
  inactive form of, 104
  liver, amphibian differentiation and, 61
  quinones and, 190
  selenium and, 185
Sucrose,
  absorption of, 373
  seed germination and, 32–33
Sugar,
  halophiles and, 137
  sulfates of, 422
Suidae,
  saliva, amylase in, 357
Sulfadiazine, acetylation of, 424
Sulfamic acid, detoxification and, 420
Sulfapyridine, acetylation of, 424
Sulfate,
  activation of, 145
  crystalline style and, 384
  enzyme repression by, 99
  methane oxidation and, 146
  molybdenum and, 184
  osmotic balance and, 138
  photosynthesis and, 142
  plants and, 137
  reduction of, 144
  sea urchin egg development and, 45
  spore germination and, 5
  surface water, origin of, 130
Sulfathiazole, acetylation of, 424
Sulfhydryl groups, keratin utilization and, 387–388
Sulfides, primitive biosphere and, 130
Sulfite,
  oxidation, 143, 145
    lipoate and, 199
  reduction of, 144
  thiosulfate formation from, 145
Sulfoconjugation, hormones and, 300
Sulfokinase(s), specificity of, 422–423
Sulfonamides,
  acetylation of, 205, 423–425, 447
  p-aminobenzoate and, 201
  folic acid deficiency and, 200
Sulfones, formation of, 409
Sulfoxides, formation of, 409
Sulfur,
  balance, maintenance of, 127
  compounds, detoxification of, 409–410
  inorganic, ruminant and, 363
  metabolism, cystine and, 153
  oxidation of, 144
  photosynthesis and, 142
Sulfuric acid, secretion of, 383
Sulfuric esters, detoxification and, 404
Sunflower,
  seed, germination of, 32
Swayback, deficiency in, 181
Swine,
  stomach, endoproteinases in, 357–358
Syllidia, neurosecretion in, 259
Symbionts,
  digestion and, 389–390
  enzyme control in, 106
  sexual cycles, ecdysone and, 277
Synaptic vesicles, acetylcholine and, 251
Syncarida, molting and, 271
Syntheses, detoxification and, 413–433

Systox, oxidation of, 410

T

Tachysaurus rugosus, stomach secretion of, 372–373
Tadpoles, respiration of, 57
Taenia, rhodanese in, 434
Talpa europaea, chitinase in, 361
Tapetum lucidum, composition of, 164, 189
Tapeworm, carbohydrate requirement of, 146
Tartrate,
  isomers, permeases for, 85
Tartrate dehydrase(s), repression of, 96
Taurine,
  cystine and, 153
  emulsifiers and, 383
Tea,
  leaves, manganese in, 182
Teart, deficiency in, 184
Teeth, fluoride and, 169, 171, 176
Tegenaria, ethereal sulfates in, 421
Teleosts,
  blood osmotic pressure of, 139
  pancreas of, 374–375
Tellurium compounds, methylation of, 426–427
Temore longicornis, skeleton of, 168
Temperature,
  digestion and, 360–361
  neurosecretion and, 269
  polymerization and, 132
  spore germination and, 5, 9, 16
  sugar absorption and, 354
Template, polysaccharide synthesis and, 162
Tenebrio,
  enzyme secretion in, 385
  ethereal sulfates in, 421
  glucosides in, 415
  juvenile hormone and, 277, 278, 280
  methylation in, 426
  nicotinamide and, 187
  succinic dehydrogenase in, 226
Tenebrio molitor,
  adrenaline in, 254
  chromatropin of, 265
  metamorphosis, respiration and, 224
Tenebrionidae,
  carnitine and, 205
  essential fatty acids and, 193
  zinc requirement of, 178
Teredo, cellulase in, 147, 386, 387
Termites,
  caste determination in, 279
  ecdysone action in, 276
  juvenile hormone effect in, 279
  pheromones of, 287–288
  wood digestion in, 387
Testudo,
  amylase, pH optimum of, 373
Testudo graeca,
  aromatic acids and, 429
  stomach secretion of, 372–373
Testudo hermanni,
  active transport in, 373
  digestive secretion of, 373
Testudo mauretanica, glucuronides in, 414
Tetraethylammonium ions, acetylcholine and, 252
Tetrahydrofolic acid, glycine formation and, 149
Tetrahymena,
  D-amino acids and, 152
  choline and, 204
  enzyme induction in, 89
  nicotinamide and, 188
  purine and pyrimidine requirement of, 166
Tetrahymena pyriformis (*geleii*),
  amino acid requirement of, 160
  carbohydrate utilization by, 147
  folic acid and, 200
  iron requirement of, 179
  juvenile hormone in, 281
  lipoate and, 199
  magnesium requirement of, 176
  potassium requirement of, 174
  vitamin B_6 and, 197
Tetramethylammonium ions, acetylcholine and, 252
Tetrapoda, intestinal surface in, 349
Tetrathionate, oxidation of, 144, 145
Thalamites, digestive enzymes of, 383
Thallium, cell growth and, 186
Theobaldia incidens,
  calcium and, 175

potassium and, 174–175
sodium requirement of, 174
Thetins, choline and, 204
Thiaminase(s), deficiency disease and, 196
Thiamine,
biosynthesis of, 195
concentration of, 170
nonenzymic catalysis by, 171
pantothenate and, 198
pyrimidine and, 164, 166
requirement for, 170, 195–196, 203
spore germination and, 7
Thiamine pyrophosphate,
decarboxylations and, 195
lipoate and, 199
β-2-Thienylalanine,
deoxyribonuclease synthesis and, 83
enzyme induction and, 83, 91
Thiobacillus denitrificans, free energy of, 144
Thiobacillus ferrooxidans, free energy of, 144
Thiobacillus thiocyanoxidans, free energy of, 144
Thiobacillus thiooxidans,
free energy of, 144
medium, pH of, 136
Thiocyanate,
formation, detoxification and, 433–434
oxidation of, 144
Thioethers, oxidation of, 409
Thioglycolic acid, insulin and, 337
Thiol groups, methylation of, 426
Thiomethyladenosine, methionine synthesis and, 153
Thioneine, tocopherol deficiency and, 192
Thioplaca, free energy of, 144
Thiorhodaceae,
enzyme induction in, 84
photosynthesis by, 142
Thiospirillopsis, free energy of, 144
Thiosulfate,
formation of, 145
oxidation of, 144
photosynthesis and, 142
reduction of, 144
rhodanese and, 434
Thiothrix, free energy of, 144
Thiourea,
derivatives, toxicity of, 410
seed germination and, 31
Threonine,
biosynthesis of, 151
cocoon spinning and, 227
decarboxylation of, 152
diapause and, 228
insulin and, 336
requirement for, 151, 159–163
synthesis, feedback and, 75–77
transamination and, 151
transport of, 351
Threonine aldolase, reversibility of, 151
Threonine deaminase, isoleucine and, 76
Threonine dehydrase,
function of, 152
induction of, 92, 93
Thymidine,
anemias and, 200
assay of, 165
enzyme activation by, 104
folic acid and, 166
Thymidylate kinase, activation of, 104, 106
Thymine,
assay of, 165
folic acid and, 166, 199
requirement for, 165
Thymine-uracil oxidase, induction of, 87
Thymus,
enzyme induction in, 92–93
juvenile hormone in, 281
Thyone briareus, juvenile hormone and, 281
Thyroid, iodine and, 177–178
Thyroid hormone, 300
evolution and, 283, 289
tunicates and, 283–284
Thyroxine,
carbamyl phosphate synthetase and, 62–63
enzyme induction and, 91, 92
tyrosine and, 156
Thysanoessa inermis, skeleton of, 168
Thysanura,
glucosides in, 415
hemolymph, ions in, 140
Tiliqua nigrolutea, stomach secretion of, 372–373

Tinca vulgaris, sugar absorption by, 354
Tineola bisellicla, keratin utilization by, 387–388
Tissue cultures, enzyme induction in, 93–94
Titanium, cell growth and, 186
Toad(s),
  aminopyrine oxidation in, 446
  aromatic oxidations in, 406
  glucuronides in, 445
  hexobarbital oxidation in, 446
  hydroosmotic activity in, 303
Tobacco,
  glycosides in, 416, 418
  nicotinic acid in, 158
Tocopherol(s), *see also* Vitamin E
  biosynthesis of, 192
  electron transport and, 191–192
  function of, 126
  isoprenoid units and, 191
Tokophrya infusionum, purine and pyrimidine requirement of, 166
Toluene, detoxification of, 404
Tomatine, sugars of, 418
Tomatoes,
  ω-aryloxyalkylcarboxylic acids and, 406
  root, thiamine and, 164
Tortoise,
  amino acid transport in, 352
  aromatic acids and, 430
  demethylation by, 411
Torula, ubiquinone of, 190
Torularhodin, vitamin A and, 206
Torulopsis,
  enzyme induction in, 90
  enzyme repression in, 98–99
Toxin,
  formation, iron and, 179
Trace elements,
  functions of, 171
  requirement for, 173–187
Trachurus, rhodanese in, 434
Tragulidae, stomach of, 361
Transamination,
  amino acid transport and, 352
  hepatopancreas and, 382
Transferase(s), detoxification and, 420
Transferrin, copper in, 180
Transforming principle, spermine and, 211
Transglycosidases, induction of, 83
Translocation, potassium and, 174
Trehalose,
  glucose absorption and, 355
  insect blood and, 141
  insects and, 231
Trematodes, Pasteur effect in, 81
Triatoma infestans,
  iron requirement of, 179
  porphyrin and, 190
Tribolium, nicotinamide and, 187
Tribolium castaneum, digestive enzymes of, 383
Tribolium confusum, amino acid requirement of, 160
1,2,4-Trichlorobenzene, formation of, 438
Trichloroethanol, detoxification of, 416, 417, 419
Trichoderma, enzyme induction in, 90
Trichomonas, ascorbate and, 194
Trichomonas columbae, sterol requirement of, 209
Trichomonas foetus,
  amino acid requirement of, 160
Trichomonas vaginalis,
  linoleate and, 193
  purine and pyrimidine requirement of, 166
Trichophyton, nicotinamide and, 188
Tridacna, digestive enzymes of, 384
Tridecapeptide, melanotropic activity of, 329
Trifolium pratense, rhodanese and, 434
Trigonelline, utilization of, 188
Trimethylamine, osmotic balance and, 138, 139
Trimethylamine oxidase, 411
Trimethylarsine, formation of, 426
Triosephosphate dehydrogenase,
  induction of, 88
  oxygen and, 80
  spores and, 15
Tripalmitin, intestinal absorption of, 356
Tripeptidase, amphibian differentiation and, 58
Triphosphopyridine nucleotide, 164
  detoxification and, 410–411

- electron transport and, 187
- glycine formation and, 149
- reduced, spores and, 18
- sea urchin eggs and, 47–49

Trithionate, oxidation of, 145
Triticum aestivum, glycosides in, 417
Triticum sativum, germination of, 35–36
Triticum spelta, rhodanese and, 434
Triticum vulgare,
- glycosides in, 417
- rhodanese in, 434

Tritonium, digestive secretion of, 383
Trout,
- *p*-amino benzoate deficiency in, 201
- glucuronyltransferase in, 420

Trutta lacustris, digestive tract of, 375
Trypanosoma cruzi,
- porphyrin and, 190
- purines and, 166

Trypanosoma evansi,
- enzymes, loss of, 389

Trypanosomidae, ascorbate and, 194
Trypsin,
- arginine-vasopressin and, 310–311
- arginine-vasotocin and, 312
- chromatotropins and, 264
- collagen and, 388
- corticotropin and, 325
- fibrin digestion by, 356
- insulin and, 336–337
- keratin and, 387
- α-melanocyte-stimulating hormone and, 321
- β-melanocyte-stimulating hormone and, 322
- reptile intestine and, 373

Tryptazan, enzyme induction and, 83
Tryptophan,
- degradation of, 158
- insect development and, 228
- nicotinamide synthesis and, 187–188
- ommochromes and, 158, 228, 233
- permease, induction of, 85
- phenylalanine and, 157
- requirement for, 157, 159–163
- riboflavin complex with, 189
- serine and, 151
- synthesis, feedback inhibition of, 76, 77
- transport of, 351

Tryptophanase, product inhibition of, 79
Tryptophan pyrrolase,
- activation of, 104, 106
- induction of, 92, 93, 94

Tryptophan synthetase,
- repression of, 95–98
- zinc and, 178

Tuberolachnis, glucosides in, 415
Tubularia crocea, juvenile hormone in, 280
Tumors,
- β-naphthylamine and, 407
- tissue culture, amino acid requirement of, 162

Tuna, pepsin of, 376
Tungstate, molybdenum deficiency and, 184
Tunicates,
- acetylcholine and, 248, 249, 251
- blood, carbon dioxide in, 139–140
- cellulose in, 163
- 5-hydroxytryptamine in, 255
- iodine fixation by, 284, 285

Turacin,
- copper in, 180
- porphyrin and, 189

Turbellaria,
- extraintestinal digestion in, 389
- juvenile hormone and, 280
- neurosecretion in, 257–258

Turkey,
- aromatic acids and, 429
- gizzard pH in, 370

Turnip, goitrogen in, 178
Turtle,
- aromatic acids and, 428, 429, 432
- detoxification in, 404
- methylation in, 425
- oxytocic activity in, 303
- vitamin A of, 206

Tyrian purple, prochromogens of, 422
Tyrosinase,
- inactive forms of, 104
- induction of, 89, 90
- repression of, 99

Tyrosine,
- arginine-vasopressin and, 310–311
- arginine-vasotocin and, 312
- enzyme repression by, 103
- insect development and, 227, 228, 229

- insect hemolymph and, 140
- α-melanocyte-stimulating hormone and, 321
- oxidation, ascorbate and, 194
- oxytocin and, 309
- phenylalanine requirement and, 156
- requirement for, 156, 159, 160, 162, 163
- sclerotization and, 234–235
- synthesis, repression of, 96

Tyrosine transaminase,
- copper and, 180
- induction of, 92, 94

U

Ubiquinone, biosynthesis of, 190–191

Uca pugilator,
- chromatotropin of, 264
- juvenile hormone in, 280

Ultraviolet,
- amino acid formation and, 130
- enzyme derepression and, 96
- enzyme induction and, 82, 89, 90
- spore germination and, 19
- vitamin D and, 209–210

Umbelliferone, detoxification of, 421

Unio, digestive enzymes of, 384

Upogebia affinis, juvenile hormone and, 280

Uracil,
- assay of, 165
- feedback inhibition by, 78
- requirement for, 165–166

Urea,
- abiogenic formation of, 131
- arginine and, 154
- aspartate formation and, 130
- hepatopancreas and, 382
- intestinal absorption of, 354
- osmotic balance and, 138, 139
- ruminant digestion and, 362–363, 365
- seed germination and, 36
- synthesis of, 61
- ureidosuccinate formation and, 130

Ureidosuccinase, manganese and, 182

Ureidosuccinate,
- abiogenic formation of, 130
- assay of, 165

Ureotelism, change to, 61

Uric acid,
- glycine and, 432
- hepatopancreas and, 382
- insect hemolymph and, 140

Uricase,
- copper and, 180
- induction of, 89, 90

Uricotelism, ornithine conjugation and, 431–432, 446

Uridine, assay of, 165

Uridine diphosphate glucose, detoxification and, 416–417, 419–420

Uridine diphosphate glucose oxidase, occurrence of, 420, 445

Uridine diphosphate glucuronic acid, detoxification and, 419, 448

Uridine triphosphate, sea urchin eggs and, 41, 42

Uridylate,
- product inhibition by, 78
- sea urchin eggs and, 41, 42

Urocanylcholine, occurrence of, 248

Urochordates,
- cellulose and, 163
- endocrinology of, 283–285

Uterus, oxytocic activity and, 302–303

Utricularia, prey capture by, 390

V

Valine,
- insulin and, 336
- mevalonate and, 208
- pantothenate and, 198
- requirement for, 152, 159–163
- synthesis, 152
  - feedback inhibition of, 76, 77
  - repression of, 95, 96
- transport of, 351

Vanadium,
- accumulation of, 185, 186
- molybdenum and, 183
- requirement for, 128, 185–186

Vanillin, detoxification of, 416

Vasopressin, purification of, 304–307

Vasopressor activity,
- assay of, 302
- mammals and, 303
- species and, 314

Vegetables, stomach action on, 360

Venoms, 5-hydroxytryptamine in, 255

Ventriculus, *see* Gizzard
Venus, habitability of, 134
Venus mercenaria,
  acetylcholine effect on, 250–253
  5-hydroxytryptamine in, 255
  juvenile hormone in, 280
Vertebrates,
  absorption of digestive products in, 349–354
  acetylcholine and, 253
  amino acid requirements of, 162
  aniline and, 407
  arginine and, 154
  aromatic hydroxylation in, 406, 409
  calcium and, 175
  cellulose digestion in, 385
  cobalt requirement of, 184–185
  copper deficiency in, 181
  detoxification in, 404
  essential fatty acid requirement of, 193–194
  eye of, 206
  heart, adrenaline and, 254
  inactive enzymes in, 104
  iodine requirement of, 178
  juvenile hormone in, 281
  land, internal pH of, 138
  lower,
    corticotropin and, 318
    insulin action in, 332
    sugar absorption by, 353–354
  manganese requirement of, 182
  monogastric, stomach and intestine of, 357–361
  mouth, digestion and, 357
  Pasteur effect in, 80
  phenothiazine and, 406–407
  phenylacetaldehyde and, 405
  phosphoramides and, 411
  potassium and, 175
  protein requirement of, 149
  purine and pyrimidine requirements of, 167
  pyrethrins and, 434
  skeletons of, 169
  special groups, digestion in, 356–377
  ω-substituted fatty acids and, 405
  sulfate conjugation in, 445
  Systox and, 410
  vitamin D requirement of, 209
Vespula carolina, synaptic vesicles of, 251
"*Vibrio aestuarii*," 137
Vibrio costicolus, salt requirement of, 137
Vicia faba,
  acetylation in, 425
  glycosides in, 416
Villi, intestinal surface and, 349
L-5-Vinyl-2-thiooxazolidone, occurrence of, 178
Viruses, origin of, 133
Vitamin(s),
  acyl group transfer and, 198–199
  carbon dioxide fixation or formation and, 195–197
  α-carbon labilization and, 197–198
  cecum and, 366
  definition of, 172–173
  electron transport and, 187–195
  single-carbon transfer and, 199–205
  ruminants and, 363
Vitamin A,
  absorption spectra of, 205
  function of, 205
  isoprenoid units and, 191
  naturally occurring forms of, 205
  oxidation of, 207
  requirement for, 172, 207–208
    quantitative, 126
  tocopherol and, 192
Vitamin B_T, *see* Carnitine
Vitamin B_6,
  functions of, 197
  polyenoic acids and, 193, 194
  requirement for, 197–198
Vitamin B_{12},
  anemias and, 200, 203
  choline and, 204
  cobalt and, 171, 184
  function of, 202
  methionine and, 153
  microorganisms and, 170
  naturally occurring forms of, 201
  porphyrin and, 189
  pyrimidine synthesis and, 102
  requirement for, 199, 202–203
  threonine and, 152
Vitamin D,
  calcium deposition and, 169

  function of, 209
  quantitative requirement for, 126
Vitamin E, *see also* Tocopherol
  classification of, 172
  deficiency,
    inositol and, 210
    selenium and, 185
  methylene blue and, 122
Vitamin(s) K,
  biosynthesis of, 191
  classification of, 172
  electron transport and, 191
  isoprenoid units and, 191
Vitamin P, function of, 211

W

Walker carcinoma cells, asparagine and, 150
Wasps, manganese in, 182
Water,
  absorption,
    cecum and, 372
    intestinal, 352–356
    omasum and, 363
    rumen and, 363
  balance,
    arginine vasotocin and, 316
    gonadotropic hormone and, 282
  Mars and, 134
  metabolism, regulation of, 139
  photolysis,
    atmosphere and, 129–130
    solar energy and, 141–142
  seed germination and, 30
  vapor, volcanic gas and, 130
  Venus and, 134
Wax, digestion of, 388
Whale,
  ethereal sulfates in, 420
  insulin of, 336
  neurohypophyseal hormones of, 307
  posterior pituitary powder, activity of, 314
Wheat,
  acetylation in, 425
  ω-aryloxyalkylcarboxylic acids and, 406
  2,4-dichlorophenoxyacetic acid and, 418
  germ, glycosides in, 416, 419
  germination of, 32, 35–36
  glycosides in, 416
  growth, silicates and, 167
  juvenile hormone in, 281
  tissues, respiration of, 148
White muscle disease, selenium and, 185
Wilson's disease, copper and, 181
Windsor bean, germination of, 32
Wood,
  digestion of, 387
  nutritive use of, 147
Woodlice, ethereal sulfates in, 421
Wood pigeon(s), benzoic acid and, 429
Worms, hormones in, 270

X

Xanthine, guanine requirement and, 166
Xanthine oxidase,
  induction of, 92
  molybdenum and, 183, 184
Xanthommatin, synthesis of, 233
Xanthophores, chromatotropins and, 263, 264
Xanthopterin, folic acid requirement and, 200
Xanthurenic acid, conjugation of, 427
Xenon, primitive biosphere and, 129
Xenopus, melanocorticotropic hormones and, 318
Xylose,
  degradation, induction of, 89
  glycoside formation and, 418
  intestinal absorption of, 349–350, 354
Xystrocera globosa, wood utilization by, 387

Y

Yeast(s), *see also* Microorganisms
  acetylation in, 425
  amino acids and, 158
  ethereal sulfates and, 422
  growth requirement of, 169, 170
  hexokinase, 79
  inactive enzymes in, 104
  iodide and, 177
  iron and, 179
  juvenile hormone in, 281
  lysine synthesis by, 155
  maltase, induction of, 93
  oxygen-induced enzymes of, 19
  Pasteur effect in, 80, 81

respiration, carbon monoxide and, 39–40
starved, amino acid pools of, 23
sterols in, 208
thiamine and, 165
vitamin A and, 206
Yolk,
formation, gonadotropic hormone and, 282

Z

Zinc,
requirement for, 178–179
replacement of, 186
spore germination and, 9
Zinc-cysteine, tapetum lucidum and, 164
Zooflagellates, amino acid requirement of, 160
Zoopherin, *see* Vitamin B_{12}
Zoospores,
morphology, lysine biosynthesis and, 124
Zymogens,
activation of, 105
digestive enzymes and, 348
enzyme induction and, 83

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